



TITLE:

Studies on proteinase inhibitors in plant seeds(Dissertation_全文)

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CITATION:

Ogawa, Tadashi. Studies on proteinase inhibitors in plant seeds. 京都大学, 1969, 農学博士

ISSUE DATE:

1969-11-24

URL:

<https://doi.org/10.14989/doctor.k948>

RIGHT:

STUDIES ON
PROTEINASE INHIBITORS IN PLANT SEEDS

TADASHI OGAWA

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ABBREVIATION

ATEE	Acetyltyrosine ethylester
BAEE	Benzoylarginine ethylester
BAPNA	Benzoylarginine paranitroanilide
TAME	Toluenesufonylarginine methylester
DFP	Diisopropylfluorophosphate
DIP-	Diisopropylphospholyl-
TNBS	Trinitrobenzenesulfonic acid
TNP-	Trinitrophenyl-
CHD	Cyclohexanedione
pCMB	p-Chloromercuribenzoate
STI	Soybean trypsin inhibitor

R-I, R-II and R-III

Inhibitors from R. sativus

B-I, B-II, B-III and B-IV

Inhibitors from B. juncea

Chapter I Introduction

Recently, many synthetic low molecular organic compounds have been known as a specific inhibitor for proteinases; DFP for a "serine proteinase" and pCMB for a "SH-proteinase". In addition to this, it is also known that there exist very unique substances, which are identified as protein, having strong inhibitory activities against certain proteinases, especially trypsin, in nature.¹⁾²⁾

Since the protein that had specific inhibitory action on trypsin was isolated from soybean by Kunitz³⁾ in 1946, a number of proteinase inhibitors have been investigated with regard to the tissues of various plants and animals.²⁾ These substances, in contrast with the synthetic inhibitors, were designated, by Laskowsky in 1954, as "the naturally occurring proteinase inhibitors" which are a class of proteins capable of combining with proteinases to form an inactive complex.¹⁾

The existence of naturally occurring proteinase inhibitors have been wide known in plant seeds. In 1946, Borchers and Ackerson⁴⁾ showed the occurrence of these substances in various legumes and, furthermore, from other seeds extracts as shown in Table I-1 many workers obtained the similar inhibitors. Some of these inhibitors have been studied in terms of the protein chemistry, especially as to the example of a reaction between two protein molecules, an

Table I-1 Occurrence of Inhibitors in Plant Seeds

Soybean	Kunitz (1946) ³⁾
Limabean	Tauber (1949) ⁵⁾
Kidney bean	Pusztai (1966) ⁶⁾
Mung bean	Sohonie (1955) ⁷⁾
Double bean	Sohonie (1955) ⁸⁾
Indian field bean	Sohonie (1955) ⁹⁾
Wheat	Shyamala (1964) ¹⁰⁾
Barley	Burger (1966) ¹¹⁾
Rye	Hochstrasser (1969) ¹⁴⁾
Corn	Hochstrasser (1967) ¹³⁾
Lettuce	Shain (1965) ¹²⁾

enzyme protein and an inhibitor protein, resulting in a forming of a third protein.¹⁵⁾¹⁶⁾¹⁷⁾

On the other hand, some of these inhibitors of plant origins were studied in connection with the nutrition of animals. The growth depression or the pancreatic hypertrophy occurring in animals fed with raw soybean meal was discussed in the matter of trypsin inhibitors present in the meal.¹⁸⁾¹⁹⁾²⁰⁾²¹⁾ Furthermore, the workers in the field of the clinical medicine took notice of those substances, which had comparatively low molecular weight and inhibited the specific enzymes (kallikrein, plasmin and thrombin), and started the research of the therapeutic possibilities or the application of those inhibitors.²⁾

Recently we demonstrated the presence of proteinase

inhibitors in several seeds of Cruciferae and others. The seeds of Cruciferae and soybean are the principal sources of the vegetable oil in Japan and sometimes used as feed after being defatted. However, in spite of the presence of these particular and interesting proteins in plant seeds as the actual components, there is a little information with regard to the protein chemistry and the nutritional problems of a reserve protein. It is, therefore, necessary to elucidate the precise nature of these proteins in order to dissolve the role of them in seeds, the specific interactions with enzymes and the behavior when used for the feed to animals.

In this investigation, the author aimed to perform the isolation and the elucidation of more precise natures about such inhibitors in order to discuss the nutritional problems of the reserve proteins, especially the protein chemical characters and the mechanism of the specific interactions with enzyme proteins.

Chapter II Distribution of Proteinase Inhibitors in Plant Seeds

As described in Chapter I, the presence of proteinase inhibitors in plant seeds were considerably reported in legumes, but except for the legume we only found their existence in wheat, rye, barley, corn and lettuce. However, taking it into consideration that the inhibitors occurred in not only a monocotyledonous seed but a dicotyledonous seed, the interest in the distribution of the inhibitors lead us to the suggestion of being possibility of the universal component of seeds as the dominant form of plants. Then the author first aimed to perform the demonstration of the presence of the inhibitors in various seeds.

1) Materials and Fundamental Methods

(i) Materials.

Various seeds were kindly supplied by Takii and Co., Ltd. Hammarsten's casein was purchased from E. Merck AG., Darmstadt. Hemoglobin was a product of Difco Lab., and acid-denatured hemoglobin solution (HCl-Hb) was prepared by the method of Schlamowitz and Peterson.²²⁾ Acetyl-L-tyrosine ethylester (ATEE), α -N-benzoyl-L-arginine ethylester (BAEE), Toluenesulfonyl-L-arginine methylester (TAME) and α -N-benzoyl-D, L-arginine-p-nitroanilide (BAPNA) were obtained from the Institute for Protein Research, Osaka University (Osaka, Japan).

Enzymes and soybean trypsin inhibitor (STI) used in this study were purchased from the following companies; STI (2x cryst.), trypsin (2 x cryst.), chymotrypsin (2 x cryst.) and pepsin (1 x cryst.) from Worthington Biochemical Co., Pronase-P* from Kaken Kagaku Co., Ltd. (Tokyo, Japan) and crystalline Nagarse** from Nagase and Co., Ltd. (Osaka, Japan).

(ii) Determination of protein concentration.

Protein concentrations were determined by the method of Lowry et al.,²³⁾ using bovine serum albumin (Armour's fraction V) as the standard and were also measured by the optical density method at 280 m μ with a Shimadzu QV-50 spectrophotometer.

(iii) Assay of proteinase activity.

The esterolytic activity for ATEE and BAEE was determined by the pH-stat method using a Radiometer model SBR2/SBUL/TTT1 a titration equipments. The reaction mixture contained enzyme, 10 mM ATEE or 10 mM BAEE and 100 mM KCl in a total volume of 2.5 ml. It was held at 25°C and kept under a nitrogen gas stream. The hydrolysis of TAME is measured by the increase in absorbancy at 247 m μ , based on the method of Hummel.²⁴⁾ Hydrolytic activity for BApNA was measured by the

* Neutral proteinase obtained from Streptomyces griceus

** Alkaline proteinase obtained from Bacillus subtilis

modified method of Erlanger.²⁵⁾ Fifty mg of BApNA were dissolved in 1 ml of dimethylsulfoxide and the solution was brought up to 100 ml with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M CaCl₂. The reaction mixture consisted of 0.2 ml of buffer, 0.2 ml of enzyme solution and 2 ml of 1.2 mM BApNA solution. After incubation for 10 min at 25° C, the reaction was stopped by an addition of 1.0 ml of 10% acetic acid and the optical density of the reaction mixture was determined at 410 mμ. Proteolytic activity was measured by the method of Hagihara et al.²⁶⁾ with microscale modification.

The incubation mixture contained 2 ml of protein solution which was composed of 1% casein or 1% HCl-Hb, 0.2 ml of buffer and 0.2 ml of enzyme solution. After incubation at 25°C for 30 min, 2 ml of trichloroacetic acid were added and the absorbancy, at 280 mμ, of the filtrate was measured.

(iv) Assay of inhibitor activity.

To determine the inhibitor activity in a given sample, a sample solution was mixed with an enzyme solution instead of the buffer as was described in the assay of proteinase activity. After preincubation for 5 min at room temperature, the substrate solution was added and the remaining activity of the enzyme was determined. The inhibitor activity was expressed as the per cent of inhibition (I) for the control assay, with the following equation;

$$I(\%) = \frac{T - T^*}{T} \times 100$$

where T^* and T are the activities of proteinase with and without the inhibitor, respectively.

2) Inhibitory Activities of the Extracts of Various Seeds.

(i) Preparation of crude extract

Ten grams of raw seeds were ground in the hand mill with a small amount of sea sand and 0.1 M NaCl solution. The resulting paste was collected and mixed with 10-fold volume (v/w) of 0.1 M NaCl solution, followed by constant stirring for one hour. The insoluble materials were removed by filtration through Hyflosupercel in a Buchner funnel or centrifugation at 8,000 r.p.m.. The obtained filtrate or supernatant was dialyzed against 0.1 M NaCl solution and then used in the following experiments without further treatments.

(ii) Inhibition behavior of crude extract

The crude extracts obtained from various seeds were incubated with various proteinase solutions and the inhibition was determined by the method as described above. Fig.II-1 shows the inhibition behavior of Raphanus sativus extract. It appeared that the extract strongly and stoichiometrically inhibited trypsin and also showed non-stoichiometrical

inhibition on other proteinases, such as chymotrypsin and Nagarse. However, pepsin and papain were not affected at all. The stoichiometrical inhibition for trypsin was observed in the extracts of all seeds used in this experiment as demonstrated in Fig. II-2.

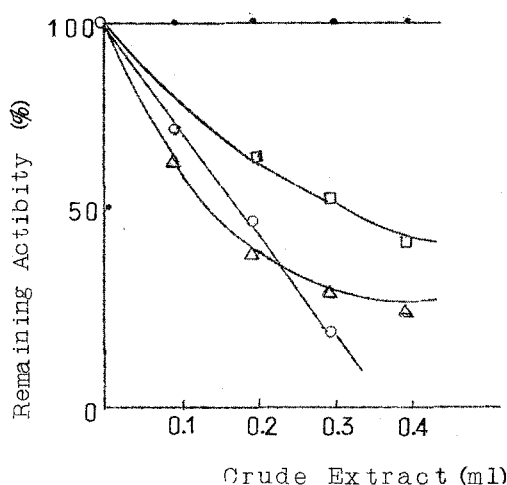


Fig. II-1 Effect of Crude Extract on Various Proteinases
 24 μ g of trypsin, 15 μ g of α -chymotrypsin,
 20 μ g of pepsin, 420 P.U.K. of Pronase and 300
 P.U.N. of Nagarse were used.

○—○ trypsin, □—□ α -chymotrypsin,
 ●—● pepsin, ○—○ Pronase,
 ▲—▲ Nagarse.

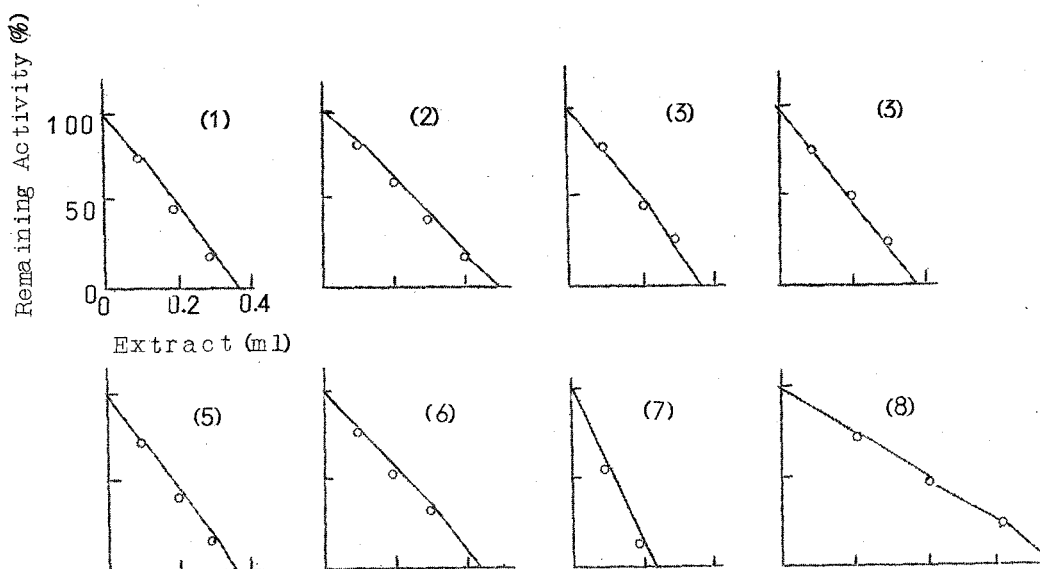


Fig. II-2 Effect of Various Extracts on Trypsin

Each extract was diluted five-fold and used for assay.

- | | |
|--|---|
| (1) <i>Raphanus sativus</i> | (2) <i>Brassica campestris</i> var. <i>nippo-oleifera</i> |
| (3) <i>B. campestris</i> Rapa | (4) <i>B. pekinensis</i> |
| (5) <i>B. campestris</i> var. <i>Komatsuna</i> | (6) <i>B. juncea</i> var. <i>rugosa</i> |
| (7) <i>B. oleracea</i> | (8) <i>B. napus</i> |

(iii) Inhibitory activity and definition of inhibitor unit

Then the inhibitor activity of each extract was determined against trypsin, for the trypsin inhibitory activity of these extracts were stoichiometrical and comparable with each other.

The International Enzyme Commission does not define the inhibitor unit in its 1961's report.²⁷⁾ We, therefore, suggest

the following definition on the trypsin inhibition; the complete inhibition of 1 μ g of trypsin is defined as one inhibitor unit. The inhibitor units of these extracts were found to be Table II-1. The values on Cruciferae seeds were comparatively higher than the other tested species. However, it appeared that the inhibitory capacities of Cruciferae were only one twentieth as strong as that of soybean obtained in the same way.

Table II-1 Inhibitor Units of Crude Extract of Various Plant Seeds

Materials	Units / g.seed
Raphanus sativus	3,360
Brassica campestris var. nippo-oleifera	2,640
B. oleracea	5,280
B. juncea var. rugosa	2,880
B. pekinensis	3,220
B. campestris var. Komatsuna	3,560
B. campestris subsp. Rapa	3,120
B. cernus	1,490
B. napus	1,560
Matthola incana	1,010
Arctium lappa	1,270
Lycopersicon esculentum	890
Solanum melongena	1,940
Sesamum indicum	940
Cucumis sativus	1,030
Spinacia oleracea	1,630

3) Gel-filtration of the Crude Extracts on Sephadex G-75

The gel-filtration was performed by using Sephadex G-75 with a column of Pharmacia (2.5 x 90 cm) and the elutions were done with 0.5 M NaCl solution. Protein concentrations were determined by the method of Lowry et al²³⁾ using bovine serum albumin as the standard and were also measured by the optical density method at 280 mμ with a Shimadzu QV-50 spectrophotometer. The patterns of the Gel-filtration were shown in Fig. II-3. These patterns were very similar to each other as to the protein elution and the inhibiting activities against trypsin. The protein fructions were designated as Fraction I, II, III and IV, respectively as shown in Fig. II-3, and the V/Vo values of inhibiting fractions, II and III were estimated to be 1.8 and 2.4, respectively.

The inhibitory activities were found separately in fraction II and III. This results led us to the suggestion that the inhibitors in these fractions were distinguishable and, in this case, there could be at least two or more different inhibitors in Cruciferae. In order to elucidate the heterogeniety of the inhibitor, the author tried to perform another experiments.

4) Electrofocusing Separation of Inhibitors

The separation of the inhibitors was performed essentially as described by Vesterberg and Svensson.²⁸⁾ An electrolysis

column of 110 ml capacity was used. It was cooled by water from a ice bath. The density gradient was made up of water solutions of sucrose and the denser solution contained 50% (w/v) of the latter component. The carrier ampholytes were

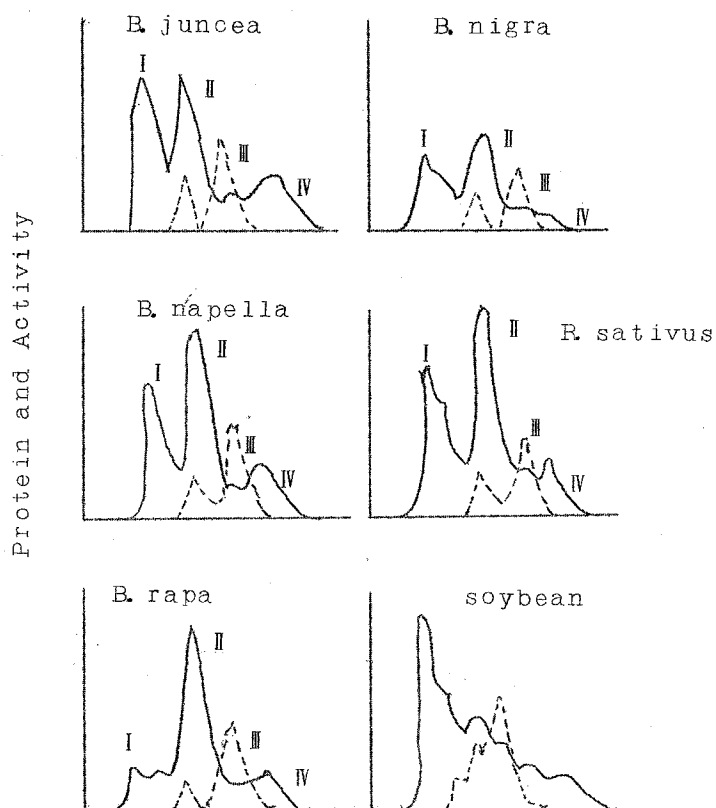


Fig. II-3 Comparison of Gel-filtration Patterns of Condensed Extracts on Sephadex G-75 Column (2.5 x 90 cm)
 _____, Protein - - - - - , Inhibiting Activity

used in order to give a pH gradient from 3 to 10. The concentration of ampholytes in the column was adjusted to 1% and the anode compartment contained sucrose (50%) to which 0.1 ml of phosphoric acid was added. The inhibitor solution obtained from the gel-filtration on Sephadex G-75 (Fraction II and III), containing 60-100 mg of protein, was dialyzed against distilled water to free it from electrolytes. The run was performed with a maximum load of 0.5 W which implies a final voltage of approximately 350 V. After focusing for 48-70 hours fractions of 2 ml were collected from the column and the inhibitory activities were determined. In Fig. II-4 the typical pattern of isoelectric separation of the inhibitor fraction obtained from Brassica juncea is shown.

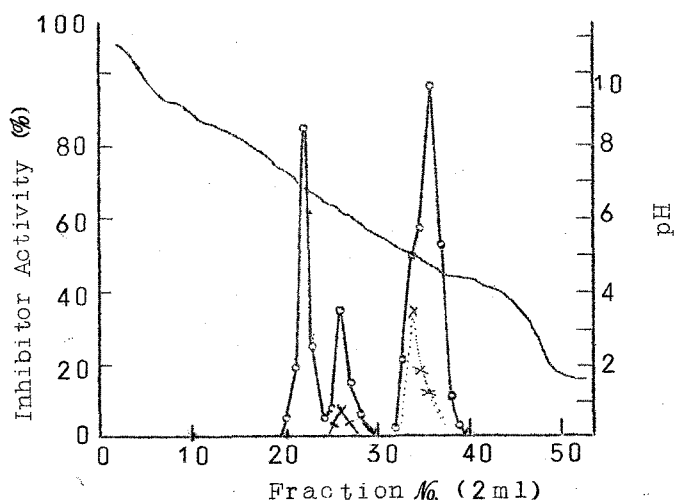


Fig. II-4 Isoelectrofocusing Pattern of B. juncea
For the determination of inhibitory activity against trypsin and chymotrypsin, 0.4 ml of each fraction was used. ○—○ trypsin inhibitor, x—x Chymotrypsin inhibition

The demonstration of the relationship between the inhibitory activities and the pH of fractions is shown in Fig. II-5.

The pI values of the inhibitors can be read off directly from this figure. Thus the results suggest that the inhibitor in the seed of B. juncea is composed of at least four different components.

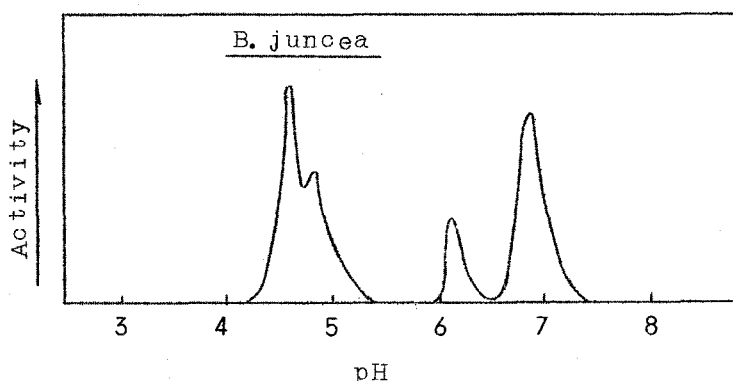


Fig. II-5 pH Diagram with Distribution of Inhibitor Activity from Isoelectric Separation

In the case of R. sativus as shown in Fig. II-6, the fraction II obtained from the gel-filtration contained an inhibitor with the pI value of 5.7 and the fraction III showed the presence of two inhibitors of pI 4.8 and 6.7, respectively. The similar observations on diversity have been also found in

soybean²⁹⁾, limabean³⁰⁾, wheat¹⁴⁾ and corn.¹³⁾ However, except for the inhibitors of soybean, they have not been identified in detail and thus the significances as to be the regular components of the seed have been also uncertain.

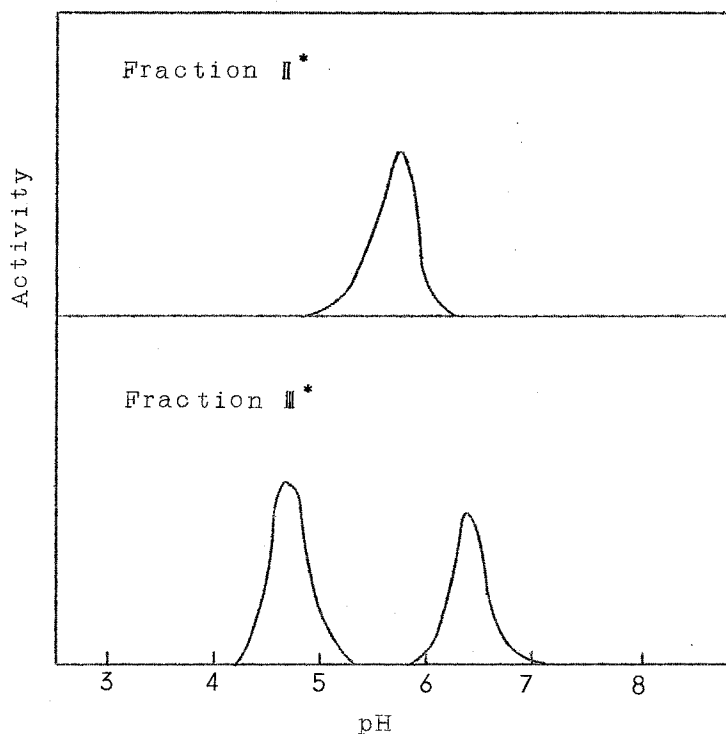


Fig. II-6 pH Diagram with Distribution of Inhibitor Activity from Isoelectric Separation
* Each fraction was obtained from the gel-filtration of crude extract on Sephadex G-75.

Chapter III Purification of inhibitors

1) Preliminary Experiments

The salting-out of the protein with ammonium sulfate is the useful method for the separation of the crude sample. Through the experiments of the salting-out of the crude extract in order to separate the inhibitors from other components the author could not get the clear results on the suitable condition of ammonium sulfate. However, the inhibiting substances were completely precipitated under the condition of more than 60% saturated ammonium sulfate. Thus the precipitate obtained from the salting-out of the crude extract was collected, dissolved in the 0.5 M NaCl solution and dialyzed in order to free from ammonium sulfate by using Visking tube. The results of the dialyzation showed the inhibiting substances escaped out slowly through the tube. (Fig. III-1) This fact suggests that the inhibitors in crude extracts consist of the comparatively low molecular substances. But the rate of the escaping through the tube was considerably slow and at the large scale preparation the loss of the yield after dialyzation may be neglected.

The following experiments on the purification were done with regard to two species of Cruciferae; R. sativus and B. juncea.

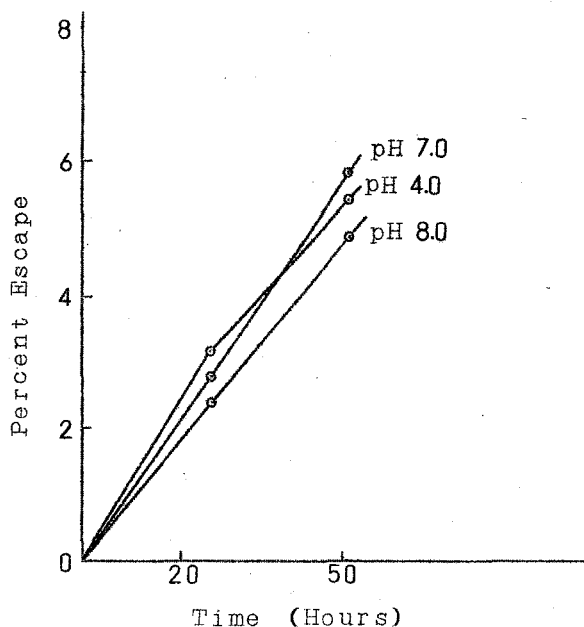


Fig. III-1 Escape Curves of Inhibitors through Visking-tube at Various pH

2) Purification of The Inhibitors from R. sativus

(i) Preparation of the crude extract.

One hundred grams of raw seed of Japanese radish were ground in a mortar with 100g of sea sand and a small amount of 0.1 M NaCl solution. The resulting paste was collected and mixed with 10-fold volume (V/W) of 0.1 M NaCl solution (1 liter), followed by constant stirring for one hour. After insoluble materials were removed by filtration through Hyflosupercel in a Buchner funnel, solid ammonium sulfate was

added up to 60% saturation (390 g/liter). The precipitate was then collected by centrifugation, at 8000 rpm for 30 min, and dissolved in 300 ml of 0.1 M NaCl solution. The solution was then heated at 80°C for 10 min in a water bath and the large amounts of precipitates produced were removed by centrifugation at 8000 rpm for 30 min. The supernatant solution was dialyzed against 0.02 M sodium acetate buffer, pH5.5, overnight and the small amount of precipitate was removed by centrifugation at 10,000 rpm for 20 min, prior to the next fractionation.

(ii) Fractionation of the crude extract.

An aliquot of the crude extract was applied to a CM-cellulose column equilibrated with 0.02 M sodium acetate buffer, pH5.5. After washing with the same buffer, elution was performed by stepwise increases of the concentration of NaCl in the same buffer; from 0.1 M to 1.0 M (Fig. III-2).

Inhibitors contained in the break-through fraction and the other two fractions were collected separately and named inhibitors R-I, R-II and R-III, respectively. Each fraction was then dialyzed overnight against 0.02 M sodium acetate buffer, pH 5.5.

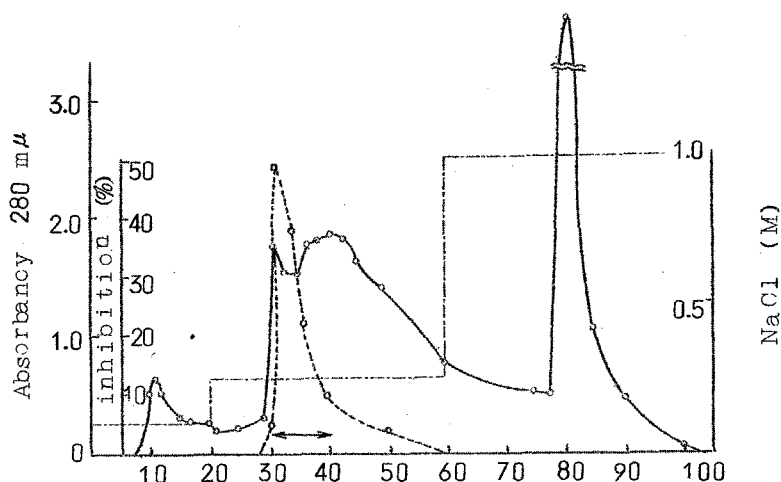


Fig. III-2 Chromatography of Crude Extract on a CM-cellulose Column (4 x 30 cm). Thirty μ l of each fraction were used for the measurement of inhibitor activity against 24 μ g of trypsin. Experimental procedures are described in the text.

—○— protein, ---○--- activity,
 ----- NaCl

(iii) Purification of inhibitor R-I.

After adjustment of the pH to 4.2 with dilute acetic acid, the dialyzate was applied to a SE-cellulose column equilibrated with 0.02 M sodium acetate buffer, pH4.2. The inhibitor was eluted by 0.02 M sodium acetate buffer, pH4.2, containing 0.1 M NaCl (Fig. III-3). The active fraction was collected, concentrated with ammonium sulfate (70% saturation) and dialyzed against 0.02 M sodium acetate buffer, pH 4.2.

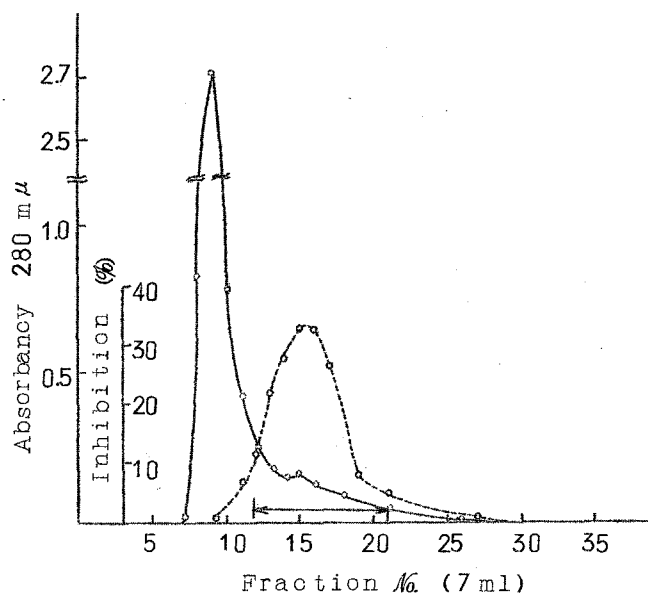


Fig. III-3 The First Chromatography of Inhibitor R-1 on a SE-cellulose Column (1.5 x 30 cm). Inhibitor activity of 10 μ l of each fraction was assayed by the same method as in Fig. II-2
 —○— protein, - - -○- - - activity

The dialyzate was applied to a SE-cellulose column under the same conditions as above. The inhibitor was then eluted with a linear gradient of NaCl, concentration from 0 to 0.1 M NaCl, in the same buffer (Fig. III-4). The active fractions were collected and used in the following experiments.

(iv) Purification of inhibitor R-II

The dialyzate was applied to a CM-cellulose column equilibrated with 0.02 M sodium acetate buffer, pH 5.5, and eluted by using a linear gradient of NaCl, concentration from 0 to 0.1 M (Fig. III-5).

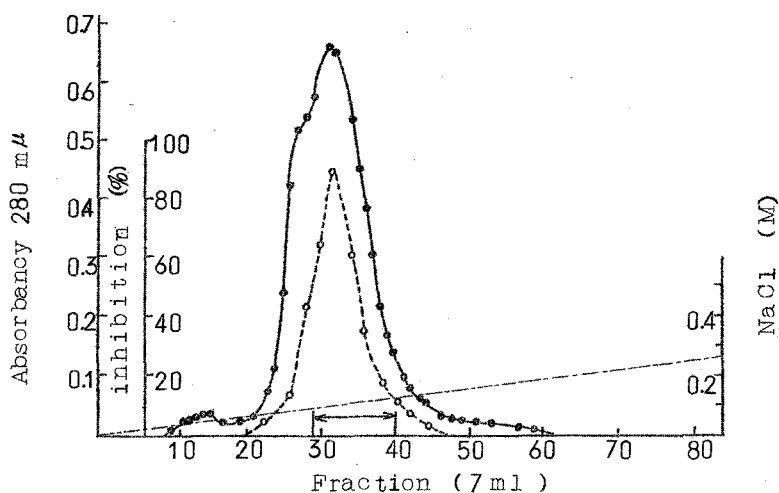


Fig. III-4 The Second Chromatography of Inhibitor R-I on a SE-cellulose Column (1.5 x 30 cm)
Inhibitor activity of 10 μ l of each fraction was assayed by the same method as in Fig. I-2
—○— protein, -○- activity,
----- NaCl

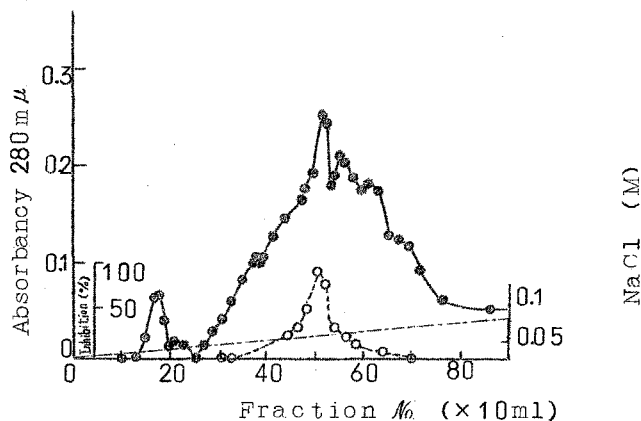


Fig. III-5 The Second Chromatography of Inhibitor R-II on a CM-cellulose Column (2 x 45 cm).
Inhibitor activity of 0.5 ml of each fraction was assayed by the same method as in Fig. I-2
—○— protein, -○- activity,
----- NaCl

The active fractions were collected and concentrated with ammonium sulfate (70% saturation). The concentrated solution, after dialysis against 0.02 M sodium acetate buffer, pH 5.5, was used in the following experiments without further purification.

(v) Purification of inhibitor R-III.

The dialyzate was chromatographed on a CM-cellulose column (Fig. III-6) and treated with the same conditions as described in (iv). The concentrated inhibitor was desalted by using a Sephadex G-15 column equilibrated with 0.02 M sodium acetate buffer, pH 5.5. The desalted solution was again chromatographed on a CM-cellulose column with a linear gradient of NaCl, concentration from 0 to 0.3 M in the same buffer, pH 5.5 (Fig. III-7). Active fractions were collected and lyophilized, after deionization by gel-filtration on Sephadex G-15.

The summary of the purification process is presented in Table III-1.

(vi) Purification of inhibitors from other origins.

It was also done by the similar procedures with slight modifications. From the extract of B. juncea the four different inhibitors were obtained, which were designated as inhibitors B-I, B-II, B-III and B-IV, respectively.

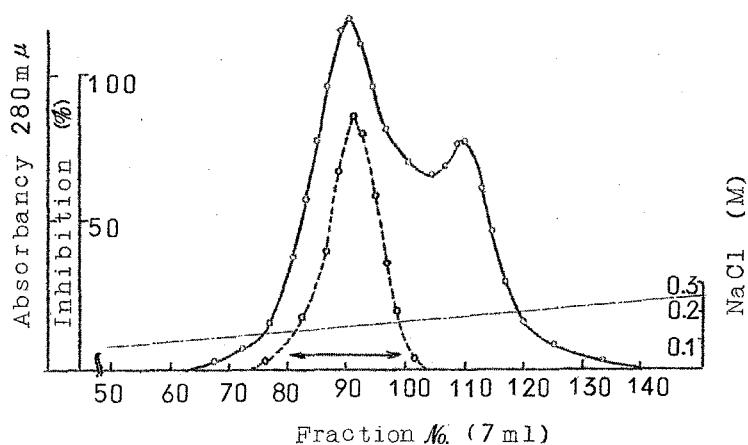


Fig. III-6 The Second Chromatography of Inhibitor R-III on a CM-cellulose Column (1.5 x 30 cm). Inhibitor activity of 10 μ l of each fraction was assayed by the same method as in Fig. II-2

—○— protein, ---○--- activity,
 ----- NaCl

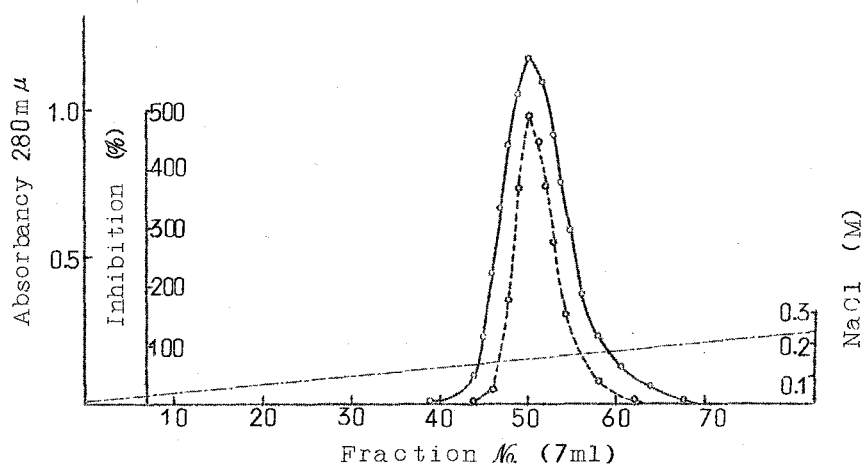


Fig. III-7 The Third Chromatography of Inhibitor R-III on a CM-cellulose Column (1.5 x 30 cm). Inhibitor activity of 10 μ l each fraction was assayed by the same method as in Fig. II-2

—○— protein, ---○--- activity,
 ----- NaCl

Table III-1 Purification of Inhibitors from R. Sativus

Raw seeds

| extracted with 0.1 M NaCl

| (NH₄)₂SO₄ 60% satu.

Ppt.

| heat treatment, 80°C, 10 min.

Sup.

| dialyzed

CM-cellulose column chromatography at pH 5.5

Fraction I (break through)

SE-cellulose column
chromatography at pH 4.2

| eluted with
0-0.25 M NaCl
(stepwise)

Rechromatography on
SE-cellulose

R.I inhibitor

Fraction II (0-0.1 M NaCl)

CM-cellulose column
chromatography at pH 5.5

| eluted with
0-0.1 M NaCl
(gradient)

R.II inhibitor

Fraction III (0.1-0.3 M NaCl)

CM-cellulose column
chromatography at pH 5.5

| eluted with
0-0.25 M NaCl
(gradient)

Rechromatography on
CM-cellulose

R.III inhibitor

Chapter IV General Properties of Purified

Inhibitors, R-I and R-III

1) Basal Properties

(i) Homogeneity: Homogeneity of the purified inhibitors was investigated by electrophoresis on cellulose acetate strip and ultracentrifugal analyses with a Spinco model E, using a synthetic boundary cell. The inhibitors showed homogeneous behaviors in these analyses, as represented in Fig. IV-1, and the sedimentation constant of inhibitor R-I was found to be 0.98 S.

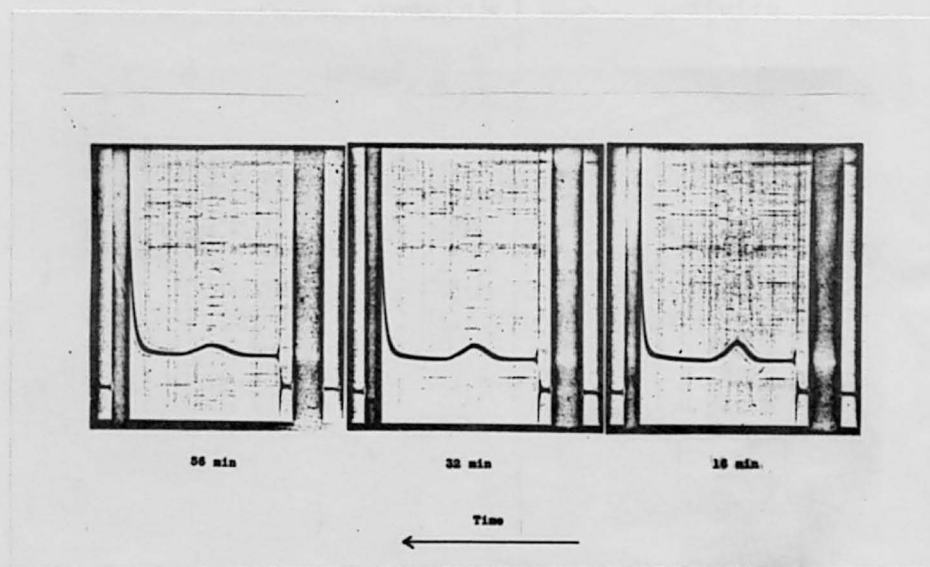


Fig. IV-1 Ultracentrifugal Patterns of Inhibitor III. Sample contained 0.4% inhibitor III, 0.1 M NaCl and 0.01 M sodium phosphate buffer, pH 7.0. Determination was carried out at 18.2°C at 59,780 rpm with synthetic boundary cell.

(ii) Ultraviolet absorption spectra and $E_{280}^{1\%}$:

The ultraviolet absorption spectra of these inhibitors were determined in 0.02 M sodium acetate buffer, pH 5.5 and typical absorption curves for protein were obtained. Absorbancies at 280 m μ of a 1% solution of inhibitors R-I and R-III in 0.05 M sodium acetate buffer, pH 5.5 were 9.80 and 9.59, respectively. (Fig. IV-2)

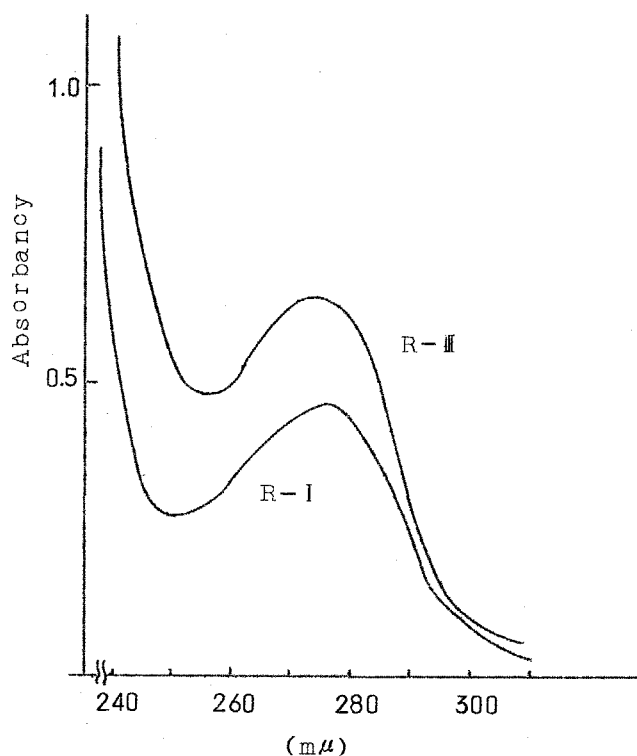


Fig. IV-2 UV Absorption Spectra of Inhibitors R-I and R-III. Experimental conditions are described in the text.

(iii) Molecular weight:

The molecular weights of inhibitors were estimated by the ultracentrifugation with the Archibald method, by the one to one molecular reaction with trypsin according to Kunitz³⁾ and by the gel-filtration on Sephadex according to Whitaker.⁵⁰⁾ It was found that the molecular weights of inhibitors R-I and R-III were approximately 8,000 and 12,000, respectively.

(iv) Nitrogen content:

The nitrogen content of inhibitor R-III was determined as 15.1% in the dry state with a Shimadzu Rapid Nitrogen Analyzer model NA-1.

(v) Isoelectric point:

The isoelectric points of these inhibitors were determined by the isoelectric focusing method as described in Chapter II. From that results it appeared that all inhibitors were an acidic protein. The pIs of inhibitors R-I and R-III were 4.6 and 6.2, respectively.

2) N-Terminal Amino Acid

The determination of N-terminal amino acid was performed essentially as described by Edman.³¹⁾

It was found that both inhibitors R-I and R-III had only valine at their N-termini.

3) Amino Acid Compositions

For the amino acid analysis the inhibitors were hydrolyzed

with 6 N HCl at 105°C in a evacuated sealed tube for 24, 50 and 72 hours, respectively. The amino acid contents of each hydrolyzate were determined using a Yanagimoto Auto Amino Acid Analyzer NC-5S. Tryptophane content was estimated spectrophotometrically according to Goodwin and Morton.³³⁾ The amino acid compositions of two inhibitors R-I and R-III are shown in Table IV-1. For the estimation of the number of amino acid residues in these inhibitors, the molecular weights of 8,000 and 12,000 were used for inhibitors R-I and R-III, respectively. The compositions of two inhibitors were very different from each other. As demonstrated in Table IV-1, in comparison with other inhibitors obtained from legumes there were no significant similarities, but histidine and methionine contents of these proteins were very low while that of glutamic acid and aspartic acid were considerably high. Cystine content of inhibitor R-III was very high as in the case of soybean 1.9 S inhibitor and limabean inhibitor. This results present interesting obserbation in terms of the structure of protein and the inhibition mechanisms.

4) Stability

(i) pH stabilities:

Aqueous samples of inhibitors at the concentrations of 50 to 100 µg per ml were doubled in volume with 0.05 M buffer

Table IV-1 Amino Acid Composition of Various Protein

	<u>Inhibitors</u> ^{a)}				
	R - I	R-III	STI ³³⁾ (Kunitz)	STI ³³⁾ (1.9 S)	LTII ³⁴⁾ (Limabean)
Lys	4	7	12	10	4
His	(0.4)	1	2	2	3
Arg	4	9	10	4	2
Asp	7	9	29	23	14
Thr ^{b)}	3	4	8	4	3
Ser ^{b)}	3	6	12	18	12
Glu	6	8	19	14	5
Pro	3	6	11	12	6
Gly	10	10	17	0	0
Ala	5	5	9	8	3
1/2 Cys	6	12	4	26	14
Val	3	2	15	2	1
Met	(0.3)	1	3	2	0
Ileu	3	2	15	4	4
Leu	3	4	15	4	3
Tyr	3	3	4	4	1
Phe	3	5	10	4	1
Try ^{c)}	0	1	2	(0.6)	0
Total	66	95	197	141	76
M. W	7,100	11,000	23,000	16,000	8,200

a) Average value from 24, 50 and 72 hour ~~by~~ drolysates

b) Extra polated value to zero hour.

c) Determined spectrophotometrically.

solutions of different pH's (from 2 to 11), and after standing for 30 min. at 37°C in the water bath, aliquots of the samples were taken and assayed for remaining inhibitory activity. The results obtained from inhibitor R-III was shown in Fig. IV-3. Under these conditions, its activity unchanged in acid pH region. However it appeared that they were comparatively affected on the activity in the region of pH 8-11.

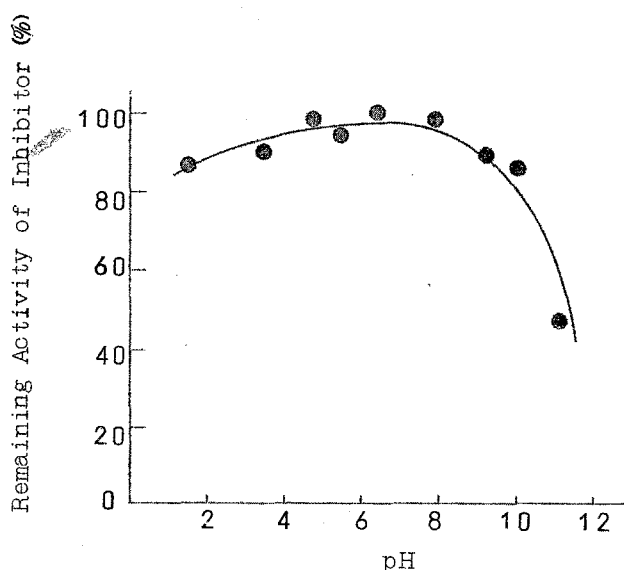


Fig. IV-3 pH-stability curve of inhibitor R-III
After incubation at various pH and 37°C for 30 min., remaining activity of inhibitor was determined.

(ii) Heat stabilities:

Inhibitors were diluted in 0.02 M sodium acetate buffer, pH 4.5 and 0.05 M Tris-HCl buffer, pH 7.5 to give a final

concentration of 220 μg per ml and preincubated to 98°C. Aliquots were removed at intervals, cooled in a ice bath and assayed for residual inhibiting activity. Stability of the purified inhibitors R-I and R-III against heating at two different pHs was tested and the results are presented in Fig. IV-4 and IV-5. R-I and R-III were very stable under conditions of heating at 98°C in the buffer of pH 4.5, however, when R-III in Tris-HCl buffer, pH 7.5 was allowed to stand at 98°C, its inhibiting activities for trypsin and chymotrypsin decreased linearly with incubation time. The discrepancy of the two curves for different enzymes suggests that different site of the inhibitor protein may participate in the inhibition of these enzymes, hence this problem will be discussed in following Chapters.

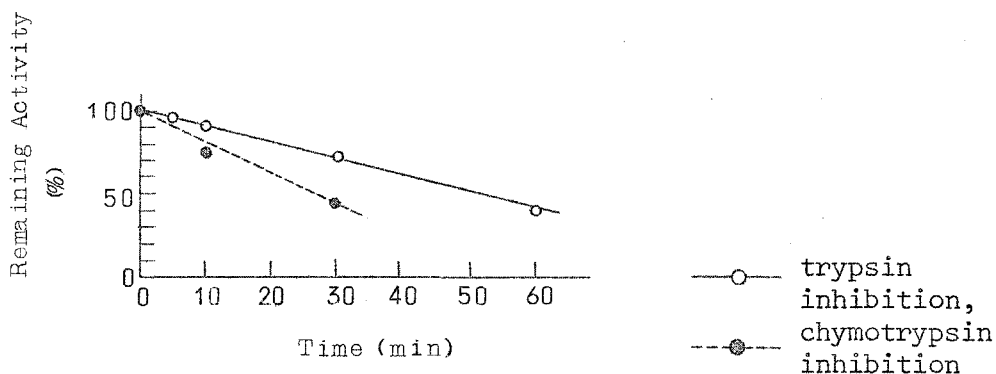


Fig. IV-4 Heat Inactivation of Inhibitor R-III
Inhibitor III was diluted in 0.05 M Tris-HCl buffer, pH 7.5, to give a final concentration of 220 $\mu\text{g}/\text{ml}$, preincubated to 98°C and assayed for the trypsin and chymotrypsin inhibiting activities by the same method. as in Fig. IV-5

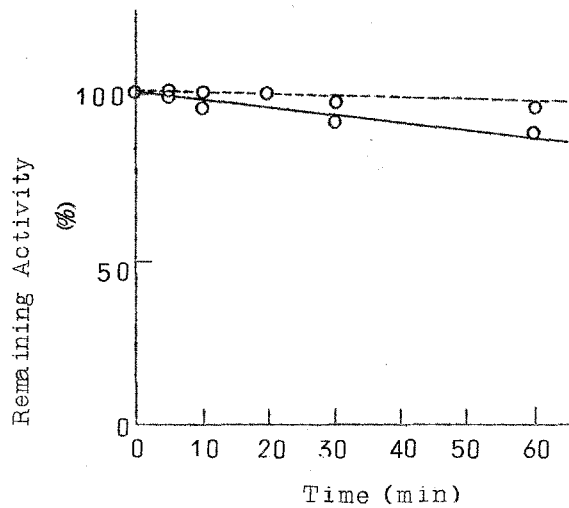


Fig. IV-5 Heat Inactivation of Inhibitors R-I and R-III
 Inhibitors R-I and R-III were diluted in 0.02 M sodium acetate buffer, pH 4.5, to give a final concentration of 220 $\mu\text{g/ml}$ and preincubated to 98°C. Aliquots were removed at intervals, cooled in an ice bath and assayed for residual trypsin inhibiting activity.
 ---○--- inhibitor R-I, —○— inhibitor R-III

Chapter V. Specificity

The extracts of R. sativus and B. juncea, as described in Chapter II, inhibited several proteinases while their capacities were different each other. In Chapter III, it appeared that the inhibitors of these extracts consisted of several different proteins. Therefore it remains still uncertain which inhibitor causes the inhibition of a certain enzyme. In order to elucidate this problem the inhibition spectra of these inhibitors were investigated. It has been reported that the inhibition spectra of inhibitors varied considerably, some were strictly specific, inhibiting only one enzyme, while others were polyvalent, inhibiting several enzymes.²⁾ On the other hand, the proteolytic enzymes generally present not only proteolysis but esterolysis. According to Ryan,³⁵⁾ it was observed that the chymotrypsin inhibitor obtained from potato inhibited the casein hydrolysis of the enzyme, whereas it showed no inhibition on the amide hydrolysis. This discrepancy seems to be caused by the use of different substrates. The activity of the inhibitor varied widely according to whether natural or synthetic substrates are used for the determination of enzyme activity. Thus, in the case of the discussion of inhibiting action, it is necessary to consider the various activities of the enzymes; that is, the various substrates which are used for the enzyme assay.

1) Trypsin Inhibition

The inhibition of trypsin activities were determined by using casein, BAEE and BApNA as the substrates of trypsin. Among various proteinases, trypsin was most powerfully inhibited by the purified inhibitors. All kinds of tryptic activities were strongly affected and also inhibited stoichiometrically

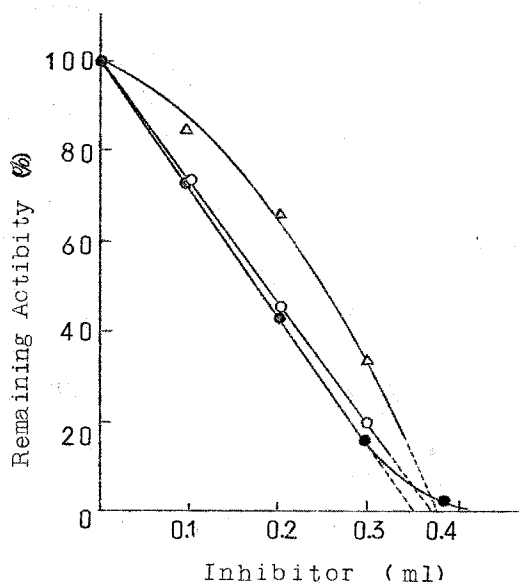


Fig. V-1 Effect of inhibitor R-III on various trypsin activities.

Substrates using in this experiment were as follows:

1% casein	—△—
1mM BApNA	—○—
10 mM BAEE	—●—

by the inhibitors (Fig. V-1) As shown in Fig. V-2, 1 μ g of inhibitor R-I completely inactivated 2.4 μ g of trypsin and 1 μ g of inhibitor R-III also inactivated 1.9 μ g of trypsin when the activity of trypsin was assayed by BApNA. The same stoichiometrical results were obtained when the substrates of trypsin were changed to BAEE or casein. Postulating that inhibitors can react with trypsin at one to one molecular ratio as in the case of the synthetic inhibitor such as DFP, the molecular weights of inhibitors R-I and R-III can be calculated as 8,000 and 12,000, respectively. These values favourably correspond to the results obtained from the estimations of molecular weights by the amino acid analysis and the ultracentrifugal analysis.

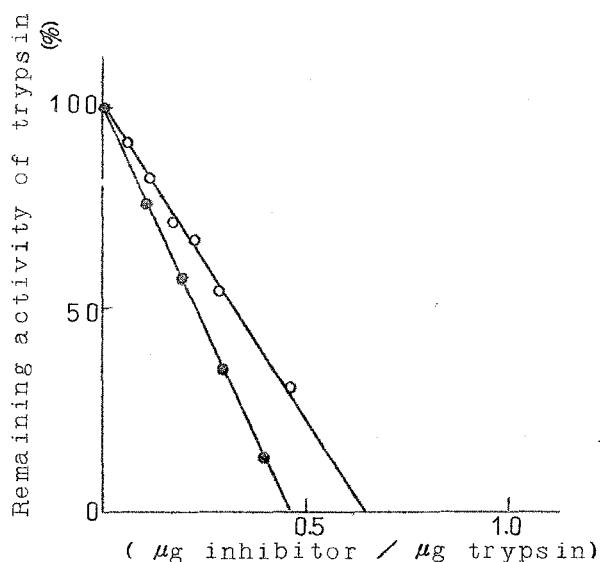


Fig. V-2 Inhibition of the Tryptic Hydrolysis of BApNA by R-I and R-III.

—●— R-I, —○— R-III

2) Chymotrypsin Inhibition

The inhibiting behaviors of the inhibitors for chymotrypsin were determined by using casein and ATEE as the substrates. It was quite different from the case of trypsin (Fig. V-3).

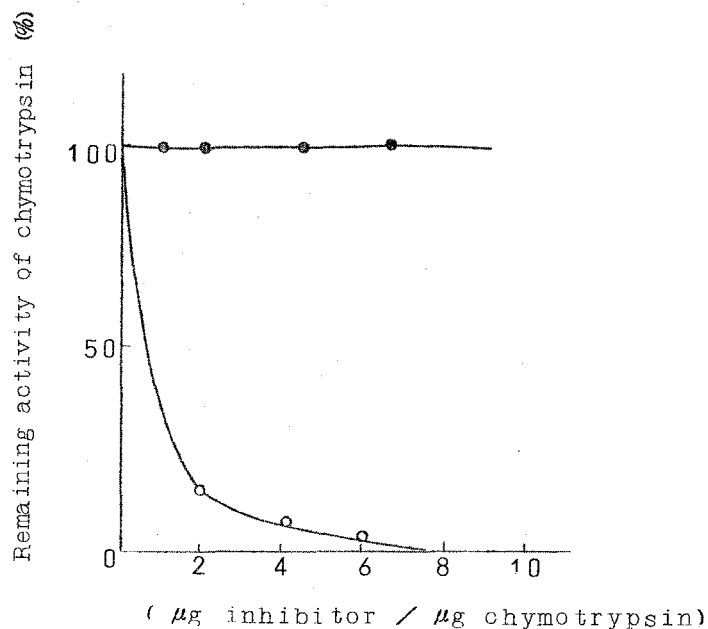


Fig. V-3 Inhibition of the Chymotryptic Hydrolysis of ATEE by Inhibitors R-I and R-III.
—●— inhibitor R-I, —○— inhibitor R-III

Inhibitor R-I was not effective on chymotrypsin in spite of increasing the amount of inhibitor added. However, chymotrypsin was considerably inhibited by inhibitor R-III, but not stoichiometrically. Also inhibitors B-I, B-II and B-III inhibited chymotrypsin non-stoichiometrically as inhibitor R-III.

3) Nagarse Inhibition

Nagarse is a chymotrypsin-like proteinase produced by *B. subtilis*. Inhibition of Nagarse was determined by using casein and ATEE as the substrates of this enzyme. As shown in Fig. V-4, a peculiar inhibiting effect on Nagarse was seen with respect to inhibitor R-III. Although R-III inhibited the ATEE hydrolysis of Nagarse, it showed no effect on the proteolysis of the enzyme.

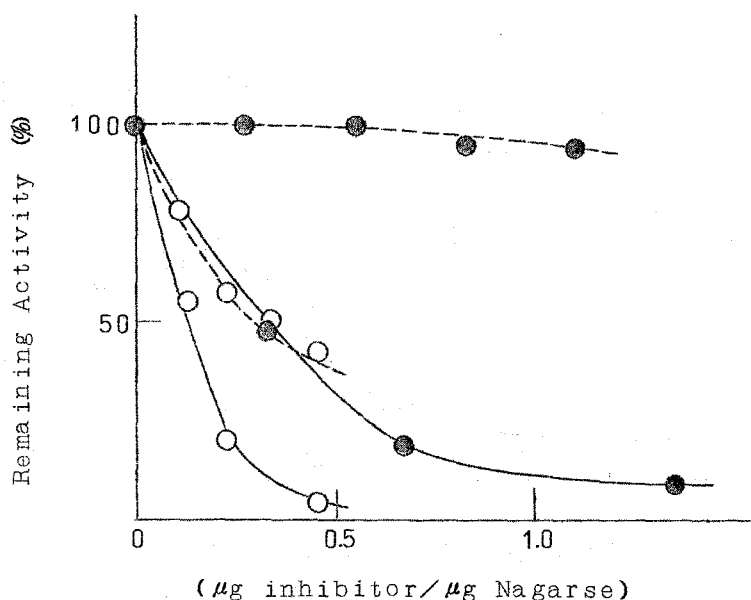


Fig. V-4 Inhibition of Various Activities of Nagarse by Inhibitors R-I and R-III. Substrates of Nagarse were 10 mM ATEE (-) or 1% casein (---).

—○— and —○— inhibitor R-I,
—●— and —●— inhibitor R-III.

On the other hand, inhibitor R-I could inhibit the hydrolysis of both ATEE and casein with Nagarse non-stoichiometrically. The results from inhibitor R-III presented the discrepancy on Nagarse inhibition, which was caused by using different substrates. It indicates that R-III can inhibit the esterase activity but not the proteolytic activity. This observation is contrast to the results of Ryan's research on the potato's chymotrypsin inhibitor which inhibits casein hydrolysis of trypsin but not its esterolytic activity.³⁵⁾ It is considered that the point of difference caused by substrates are related to the affinity of the enzyme to these substrates, if the inhibitor protein competes with the substrate. Postulating that ATEE is not suitable substrate for Nagarse rather than casein, the hydrolysis of ATEE can be inhibited more strongly than casein hydrolysis as a matter of course. The competition between a substrate and an inhibitor will be discussed in Chapter VI.

4) Inhibition of Other Proteinases

The inhibition of other proteinases, such as Pronase, Pepsin and Papain, was also determined. Pepsin and papain were not inhibited at all, but the BAPNA hydrolysis activity of Pronase was inhibited powerfully and stoichiometrically as well as trypsin. From the results described in 1), 2), 3) and 4), it has been appeared that the proteinases which were

affected by the inhibitors belong to group a "serine enzyme", which can be inhibited by DFP. These facts, therefore, present very interesting and important problems; their can exist a similar kinds of structures among these enzyme molecules. It can be considered the application of the inhibitor proteins are a valid means for analysis of the conformational similarity of these enzymes.

5) Characterization of Polyvalent Inhibition

Many inhibitors obtained from various legumes have been characterized as a "polyvalent" inhibitor.²⁾ Kunitz's soybean trypsin inhibitor has not only a capacity of trypsin inhibition but also that of cymotrypsin. According to Sohonie, the inhibitor from Broad bean inhibited not only trypsin, but also chymotrypsin and papain,³⁶⁾ as well as *Aspergillus* proteinase³⁷⁾ stoichiometrically and reversively. On the other hand, Feeney represented the term - "multi-headed type inhibition" - for some of these polyvalent inhibitors.³⁸⁾ This presentation contains two meanings. One is the case of the multiplicity of inhibition which occurs in Kindney bean's inhibitor obserbed by Pusztai,³⁹⁾ that is, the chymotrypsin inhibition by Kidney bean's inhibitor occurred in two stage, at low enzyme concentration a 1 : 1 complex formed while a high enzyme concentration more chymotrypsin reacted in a non-stoichiometric manner. The other is the case

of inhibitor strictly having a "multi-head" on its molecule, that is, for the example of Feeney's lima bean inhibitor Fract. 6, the inhibitor-trypsin complex has still chymotrypsin inhibiting capacity and the inhibitory sites for these enzymes exist independently on the inhibitor protein.³⁰⁾

In order to test for the multiplicity of inhibitors obtained from R. sativus, the following experiments were performed. Two μg of chymotrypsin and 8.2 μg of inhibitor R-III mixed in 0.2 ml of 0.01 M phosphate buffer, pH 7.5 and preincubated for 5 min at 25°C, and then the mixture was assayed with ATEE as the substrate. As shown in Table V-1, the chymotryptic hydrolysis of ATEE was reduced to 0.18 $\mu\text{moles/min/ml}$ in comparison with the activity of 3.40 $\mu\text{moles/min/ml}$ in the absence of inhibitor. However, when the mixture of 20 μg of trypsin and 8.2 μg of inhibitor R-III were added and incubated, the ATEE hydrolysis of chymotrypsin was not affected. The similar results were also obtained in the case of the reverse of experiment order. Moreover, in the case of inhibitor R-I, the similar observations were obtained with respect to Nagarse inhibition. As is mentioned above, the inhibition of trypsin with various inhibitors were stoichiometric and were only formed to react at 1 ; 1 molecular ratio. It appears, therefore, that the inhibitor is not multi-headed type but can be simply concluded to polyvalent inhibitors.

Table V-1 Inhibition Properties of InhibitorsR-I and III

Assay Conditions	Residual Enzyme Activity* (μ moles/min/ml)
Chymotrypsin (2.0 μ g)	3.40
+Inhibitor III (8.2 μ g)	0.18
+Mixture of trypsin (20 μ g) and inhibitor III (8.2 μ g)	3.15
Trypsin (10 μ g)	1.21
+Inhibitor III (20 μ g)	0.06
+Mixture of chymotrypsin (20 μ g) and inhibitor III (20 μ g)	1.34
Trypsin (10 μ g)	1.21
+Inhibitor I (9.0 μ g)	0.06
+Mixture of nagarse (6.0 μ g) and inhibitor I (9.0 μ g)	0.95

* Enzyme activities were measured by pH-Stat method using 10 mM ATEE and 10 mM BAEE for the substrates of chymotrypsin and trypsin, respectively.

Chapter VI Characterization of Inhibiting Mechanism

Recently, the inhibition mechanisms were investigated in detail with regard to the soybean trypsin inhibitor (STI) and trypsin interaction.⁴⁰⁾ This interaction was adopted as a model for the inhibition of proteolytic enzyme in general. It has been appeared that STI reacted and formed a 1 : 1 enzyme inhibitor complex with trypsin and the complex was considerably stable under the condition of enzyme assay while the reaction was pH-dependent in an acidic pH region.⁴¹⁾ The equilibrium between the trypsin and inhibitor and the complex is reached so quickly that the time course of the reaction (half-time of reaction is assumed to be less than 5 sec.) cannot be followed by any of the usual analytical methods and the dissociation constant of the complex is also determined hardly because of the large difference between the dissociation constant of the enzyme-substrate complex, $\sim 10^{-2}$ M and the enzyme-inhibitor complex, $\sim 10^{-10}$ M.²⁾ Therefore, the inhibition has been assumed to be a non-competitive type. Since, however, Green, who used the esterolytic cleavage of BAEE for the detection of trypsin activity, was the first to recognize the competitive nature of the complex,⁴²⁾ the fact that the competitive inhibition occurred led us to the conclusion that the inhibitor mainly became attached to the active center of

the enzyme. This was supported by the observation that the binding of the inhibitor no longer took place when the active site of trypsin was blocked by DFP.¹⁾ On the other hand, Ozawa and Laskowski, Jr. prepared the modified STI which once reacted with trypsin and observed that the modified STI consisted of two peptide chains joined by S-S bridges.⁴⁰⁾ They, therefore, concluded that the inhibitor hydrolyzed limitedly at the specific peptide linkage by trypsin when it formed the complex. This specific site was named as active center of the inhibitor. In the case of STI, it is a specific Arginyl (64) - Isoleucine (65) bond in the peptide chain. In addition, Feeney et al reported that the activities of limabean inhibitor were reduced when the free amino groups of lysyl residues were modified with trinitrobenzenesulfonic acid.³⁰⁾ From these results, it is expected that the inhibitor has its own active site which reacts with the active center of trypsin. Moreover, it must be arginyl or lysyl residues because of the tryptic affinity corresponding to these residues of peptide chain.

In this Chapter, the author described mainly the interaction of the trypsin and inhibitor R-III, which are well clarified as to the physicochemical properties, in order to discuss the mechanisms of the complex formation.

1) Kinetics

(i) Dissociation constant and free energy

In the pH region of optimal enzyme activity (pH 7-8) the dissociation constant can be estimated from the curvature of the plot of trypsin inhibited versus inhibitor added, near the point of neutralization. In this case, the enzyme activity measurement should be sensitive to extremely low concentration of enzyme. Therefore, the enzyme assay was performed by using a pH-Stat method with BAEE as the substrate. As shown in Fig. VI-1, the trypsin which was incubated with the equimolar quantities of inhibitor R-III presented the weak hydrolysis of BAEE which was caused by the trypsin dissociated from the complex at pH 8.0. The affinity of an inhibitor for an enzyme is usually expressed by the dissociation constant. This is defined as the inhibitor constant, K_i :

$$K_i = \frac{[E][I]}{[EI]}, \quad E + I \rightleftharpoons EI$$

where the quantities $[EI]$, $[E]$ and $[I]$ are the equilibrium concentration (mole/liter) of the trypsin-inhibitor complex, free trypsin and free inhibitor, respectively. Then the residual trypsin activity at the presence of one equivalent of

inhibitor was converted to the value of enzyme concentration. At pH 8.0, as shown in Fig. VI-1, the residual activity of trypsin was found to be 4.6 % of the full enzyme activity. The concentration of the trypsin and the inhibitor R-III used in this experiment were 3.67×10^{-7} mole/liter, respectively. Then the dissociated trypsin was calculated to be 1.69×10^{-8} mole/liter. Therefore, K_i was evaluated as follows;

$$K_i = \frac{(1.69 \times 10^{-8}) (1.69 \times 10^{-8})}{(3.67 - 1.69) \times 10^{-7}} = 8.2 \times 10^{-10} \text{ M.}$$

In the same way, the dissociation constant of the trypsin-inhibitor R-I complex and the trypsin-STI complex were obtained as $1.37 \times 10^{-10} \text{ M}$ and $8.9 \times 10^{-10} \text{ M}$, respectively. These values are compared favorably with the value of the trypsin-STI complex, $2.0 \times 10^{-10} \text{ M}$, as reported by Green.⁴³⁾

The free energy of association can be also evaluated from the equilibrium constant, K_{assoc} , for the association reaction by the use of the usual equation,

$$\Delta G^{\circ}_{\text{assoc}} = -RT \ln K_{\text{assoc}},$$

$$\text{where } K_{\text{assoc}} = \frac{1}{K_i} = \frac{[EI]}{[E][I]}$$

Therefore, the free energy of association is calculated as -12.4 Kcal/mole with respect to the trypsin-R-III complex formation.

The K_i values and the free energy of association obtained from other inhibitors are summarized in Table VI-1.

Table VI-1 Kinetic Constants of Trypsin-Inhibitor Complex

	R-I	R-III	STI	TNP-R-I
k (l·mole ⁻¹ sec ⁻¹)	5.8×10^6	1.1×10^6	8.2×10^6	3.0×10^6
$K_{\text{assoc.}}$	3.7×10^9	1.2×10^9	1.1×10^9	-
$-\Delta G^\circ_{\text{assoc.}}$ (Kcal/mole)	13.1	12.4	12.3	-

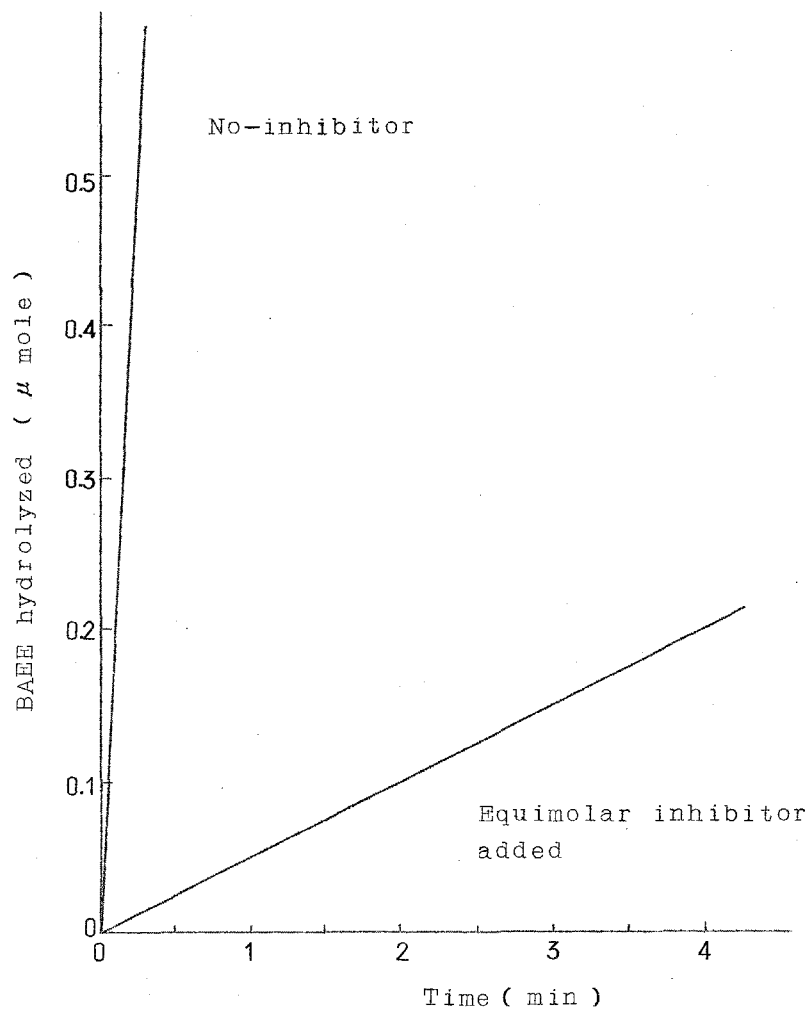


Fig. VI-1 pH-Stat Measurement of Trypsin Activity in the presence or Absence of Inhibitor R-III
The enzyme concentration used in this assay was $3.67 \times 10^{-7}M$, and the activity measurement was started after the incubation with inhibitor for 1 min.

(ii) Reaction velocity

The determination of the apparent reaction velocity of the trypsin-inhibitor complex formation was essentially the same as described by Haynes and Feeney.⁴⁴⁾ The general procedure was as follows. HCl (0.001 M) (0.2 ml) containing the enzyme was pipetted into a quartz cuvet. Then 2.6 ml of assay buffer containing an equimolar amount of inhibitor was added rapidly with vigorous mixing, and the mixture was incubated for various periods of time. At the end of the incubation period, 0.2 ml of the substrate (TAME) solution was added rapidly and with vigorous mixing to ensure complete mixing. This almost completely prevented further formation of complex during the assay period. The change with time in absorbance of the solution at 247 mμ was measured with a Shimadzu QV-50 spectrophotometer. The slope of plotted line, when compared with that for the same amount of enzyme in the absence of inhibitor, reflects the proportion of the enzyme which was not inhibited during the incubation period. To study the effect of viscosity and salt concentration on the rates of inhibition, several rates were also determined in the presence of 16 % sucrose and 1.25 M KCl.

The rates at which trypsin is inhibited by inhibitor R-III are given in Fig. IV-2 and it appears to conform to

second-order kinetics. The estimated second-order rate constants of various inhibitors are summarized in Table VI-1.

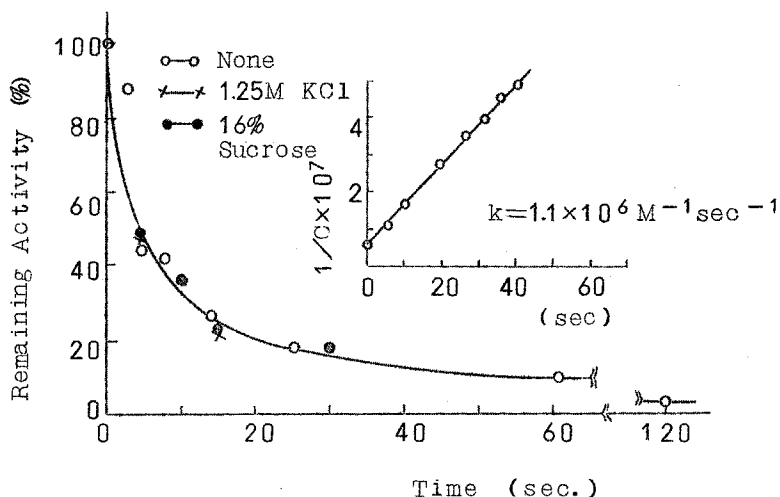
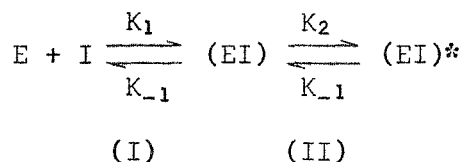


Fig. VI-2 Time Course for the Inhibition of Trypsin by Equimolar Quantity of Inhibitor (R-III)
In these assay, the inhibitors and the trypsin are incubated together for the times indicated and then substrate was added rapidly and the initial enzyme activity was determined.

In this investigation, soybean trypsin inhibitor, limabean inhibitor and TNP-R-I* were included for comparative purposes, which was reported by Haynes and Feency.⁴⁴⁾ The rate constant for the inhibition of trypsin with R-III was calculated as $1.4 \times 10^6 \text{ l. mole}^{-1} \text{ sec}^{-1}$. This value was similar to that of other inhibitors in the order of $10^6 \text{ l. mole}^{-1} \text{ sec}^{-1}$. On the other hand, no significant differences were observed when the rate of inhibition were determined in the presence or in the absence of 16 % sucrose and 1.25 M KCl, respectively,

as shown in Fig. VI-2. This observation was favorably agree with the results as reported by Haynes and Feeney,⁴⁴⁾ while the results in the presence of 1.25 M KCl differed from Green's results.⁴³⁾ The observation that the rate of inhibition was apparently independent of the viscosity of the medium indicated that the rate limiting step is not the formation of the initial enzyme-inhibitor complex, but rather is another "monomolecular" process. Therefore, the author also proposed, according to Haynes and Feeney, the simplest equation which fits these results,



where $k_1 \gg k_2 \gg k_{-2}$, (EI) is the intermediate, unstable complex, (EI)* is the stable complex which has low k_{-1} value and step II is the monomolecular, rate-limiting process which is not affected by the condition of the medium. However, whether the completely inhibited complex is modified as reported by Ozawa and Laskowski,⁴⁰⁾ or not still remains unanswered.

2) Displacement and Reversibility

In this experiment, the reaction rate was determined in

the presence of a substrate. The procedure named as "displacement method" is essentially based on the report of Green⁴²⁾ and the most direct way to test the effect of substrate on the inhibitor enzyme combination. It is a reversal of the usual procedure; to allow the trypsin and substrate to combine before addition of the inhibitor. The results of such experiments, in which various concentrations of inhibitor and substrate were employed, are shown in Fig. VI-3. It appeared that the velocity of displacement of substrate by inhibitor was greatly affected by the concentration of substrate or inhibitor. In the absence of inhibitor the usual zero order rate law was obeyed, as indicated by the straight line. The initial slope of these curves were close to this line, but all decreased with time until the equilibrium was completed. This result appeared to conform to pseudo first order kinetics as shown in Fig. VI-4. The first order rate constant of displacement of BAEE by inhibitor R-III was evaluated approximately to be 0.23 min.^{-1} at the presence of 5 mM BAEE, while it became slower when the substrate concentration was increased. The reverse of the above phenomenon was observed in the presence of higher concentration of substrate (10 mM 100 mM). It was found during such esterase runs that there was slow increase of velocity with time, indicating displacement of

inhibitor by substrate. The results of such experiments, with equivalent quantities of inhibitor and trypsin, are shown in Fig. VI-5.

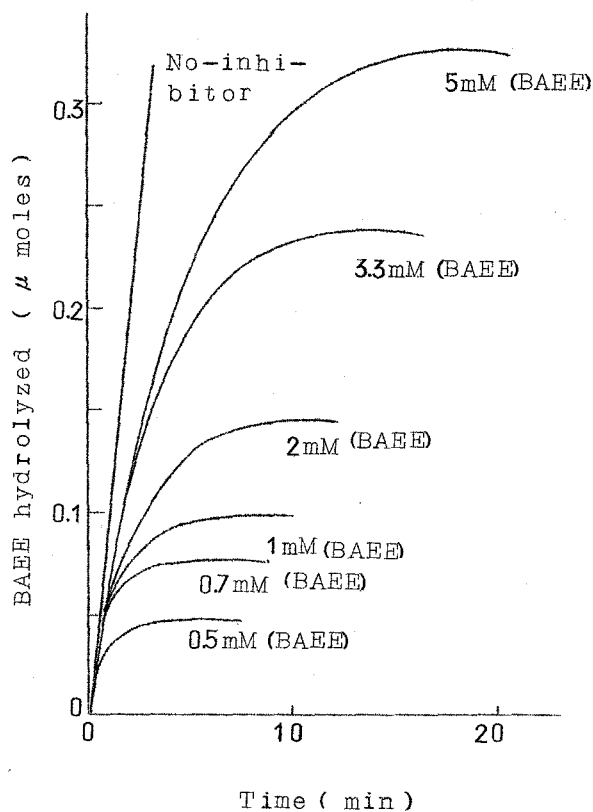


Fig. VI-3 Displacement of BAEE from Trypsin by Inhibitor, as shown by Decrease of the Reaction Velocity with time. Trypsin; 6.12×10^{-8} M, inhibitor; 1.94×10^{-6} M were used in this assay and inhibitor added last to trypsin + substrate. It was found that this order of mixing gave the same results as the final addition of trypsin to inhibitor + substrate.

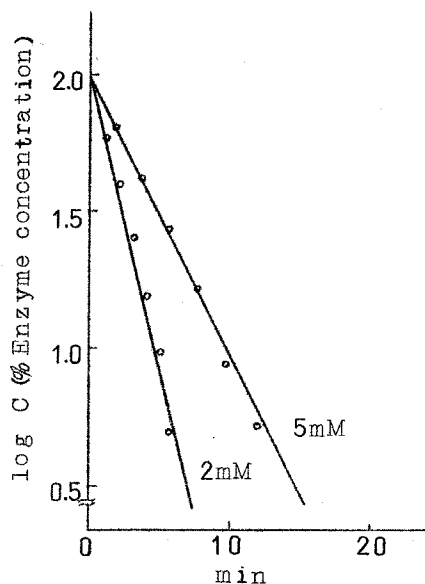


Fig. VI-4 The First Order Kinetics of the Rate of Displacement.
Experimental conditions were the same as Fig. VI-3.

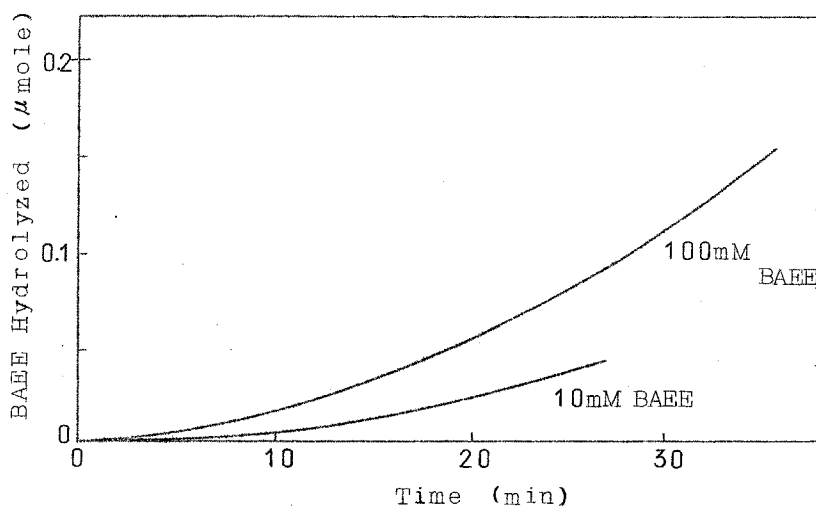


Fig. VI-5 Displacement of Inhibitor from Trypsin by BAEE, as shown by Increase of the Reaction Velocity with Time.
The substrate was added after inhibitor and trypsin had combined. The equimolar trypsin and inhibitor 3×10^{-7} M were used.

These results presented above indicate that the inhibition of trypsin by the protein inhibitors should be regarded as "competitive" and "reversible", although it differs from the usual type of competitive inhibition in the following respect; the dissociation constants of the enzyme-inhibitor complexes are so low (10^{-10} M), that the assumption on which the Lineweaver-Burk relations between reciprocal velocity and reciprocal substrate concentration are based does not apply. Similar case has been described with respect to DFP-trypsin interaction, but it strictly differs from the system of the protein inhibitor, in the point of being irreversible.

3) Interaction with Inactive Trypsin

In order to test the necessity of the active site of trypsin the DFP-inactivated trypsin was used for the assay of inhibition. The DIP-trypsin was prepared as follows; 0.7 ml of 0.05M Tris-HCl buffer, pH 7,5 and 0.3 ml of 10 mM DFP solution were added to 1 ml of trypsin solution containing 2mg of crystalline trypsin and the mixture was incubated for 5 hours at room temperature. After the incubation it was dialyzed against distilled water for 2 days at 5°C. The same methos as described in the experiment of inhibition specificity was employed in this case. As shown in Table VI-2 it is found that the inactivated trypsin can not form the

inactive complex with the inhibitors. The similar observation was also found in the case that the heat-inactivated (denatured) trypsin was used for the assay. These results, hence, indicate that the active site of trypsin participates in the interaction between the trypsin and inhibitors and, in addition, the strict conformation of enzyme should be essential.

Table VI-2 Interaction between DIP-trypsin and R-III.

Assay condition	Residual* Enzyme activity (μ moles/min/ml)
DIP-Trypsin (87 μ g)	0.11
Trypsin (40 μ g)	4.90
+DIP trypsin (87 μ g)	5.04
+Inhibitor III (20 μ g)	0.26
+Mixture of DIP trypsin (87 μ g) and inhibitor III (20 μ g)	0.28

* Enzyme activity were measured by pH-Stat method using 10 mM BAEE for the substrate of trypsin.

4) Reacting Groups

(i) Determination and modification of free amino groups

The determination and modification of free amino groups of inhibitor protein were done according to the method

of Habeeb.⁴⁵⁾ To 1 ml of protein solution (0.2 - 0.5 mg/ml) were added 1 ml of 0.5 M NaHCO₃, pH 8.5 and 1 ml of 0.1 % TNBS. The mixture was allowed to react at 37°C. After given periods the reaction was stopped by the addition of 1 ml of 10 % SDS and 1N-HCl and the absorbance of the solution was read at 335 mμ using a Shimadzu QV-50 spectrophotometer. At the same time a portion of the reaction mixture was withdrawn for the detection of inhibitor activity before termination of the reaction. Absorbancy of the reaction mixture was read at 335 mμ and average value of 1.1×10^4 given by Haynes and Feeny⁴⁴⁾ was used for the molar extinction coefficient of trinitrophenyl groups in protein. The free amino groups of inhibitors reacting with TNBS were found to correspond to the concentration of TNBS. However, under the condition described above all free amino groups could be determined and calculated. The time course of reactions were shown in Fig. VI-6. It was found that about forty minutes were required for the complete reaction when the amount of protein were less than 0.1 mg. The estimated values of free amino groups were approximately in agreement with the amino acid analysis. By the TNBS method they were calculated to be 7.5 and 5.2 per molecule with respect to R-I and -RIII, respectively while according to the amino acid analysis they were 8 and 5, respectively. Therefore, under the described condition it

appeared that all amino groups in these proteins could be modified into TNP-amino residues. The modification were done with respect to reaction time and at given period the residual activities of inhibitors were tested against trypsin and chmotrypsin. The extent of the modification and the change of activity are shown in Fig. VI-7. The inhibitory activity of inhibitor R-III against trypsin and chymotrypsin were reduced by the modification of free amino groups, whereas two curves were not parallel. The anti-trypsin activity of R-III decreased faster than the anti-chymotrypsin activity. Nevertheless, the TNBS modification of inhibitor R-I and soybean trypsin inhibitor have no effect on their activity in spite of the complete modification of their free amino groups.

(ii) Modification of arginyl redises

1, 2-cyclohexanedione is known as a reagent for modification of guanidino groups of arginyl residues in protein. The method of modification was essentially performed according to Haynes and Feeney.⁴⁴⁾ It was done by incubating the protein (ca. 1 mg/ml) in a solution containing 0.1 M triethylamine, 0.01 M EDTA, and 0.016 M 1,2-cyclohexanedione (pH 10.5) for 12 hr at room temperature. The sample were then dialyzed against several changes of deionized water.

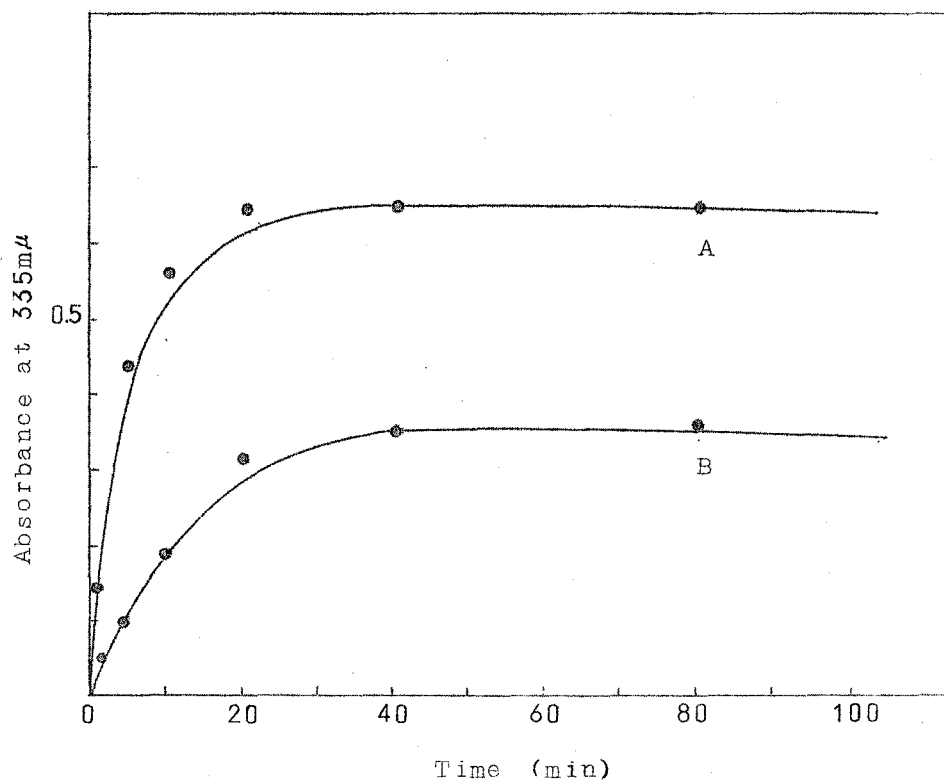


Fig. VI-6 Time Course of TNBS Modification.
Experimental procedure are described in the text.
A: 42.6 μg of R-III/ ml of reaction mixture.
B: 21.3 μg of R-III/ ml of reaction mixture.

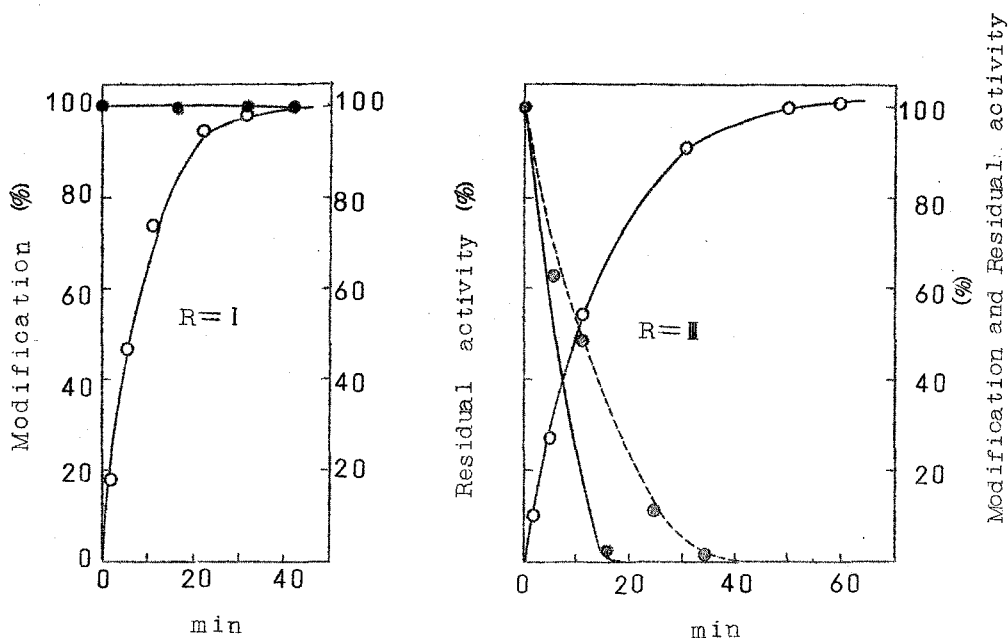


Fig. VI-7 Time course of Modification of Amino Groups with TNBS and Loss of Inhibitory Activity.

After various periods of incubation, the reaction was stopped. The number of amino groups which had been modified was determined spectrophotometrically, and assayed for residual anti-tryptic and anti-chymotryptic activity.

- modification degree
- anti-tryptic activity
- anti-chymotryptic activity.

Table VI-3 Effect of Arginine Modification on Inhibitor Activity.

Inhibitor Activity (%)	R-I	R-I	R-III	R-III
	+CHD	-CHD	+CHD	-CHD
Before Modification	100	100	100	100
After Modification	35.5	98	13.0 *	97

Modification procedures were described in the text.

* Decrease of R-III activity was caused by the destruction of lysyl-residues with CHD, that was appeared in Fig. VI-8.

As shown in Table VI-3, the activity of R-I was reduced extensively by modification of arginyl residues with CHD. However, it is reported that CHD reacts not only with arginyl residues but with lysyl residues. Therefore, it was observed that the activity of R-III was greatly affected. The degree of the modification of lysyl residues in protein was checked photometrically at 440 mμ according to Haynes.⁴⁴⁾ As shown in Fig. VI-8, the spectrum of the reaction mixture indicated the occurrence of CHD-lysyl residue reacting products.

According to the report of Liu et al,⁴⁷⁾ trypsin inhibitors may be classified as either arginine inhibitors

or lysine inhibitors. From the results of the modification, it appeared that inhibitor R-I is an arginine type and inhibitor R-III is a lysine type inhibitor. With respect to the other inhibitors, the types of inhibitors are summarized in Table VI-4. However, in the present study, it was not clear whether these functional amino acid residues in protein were specific one or not as described by Ozawa and Laskowski.⁴⁰⁾

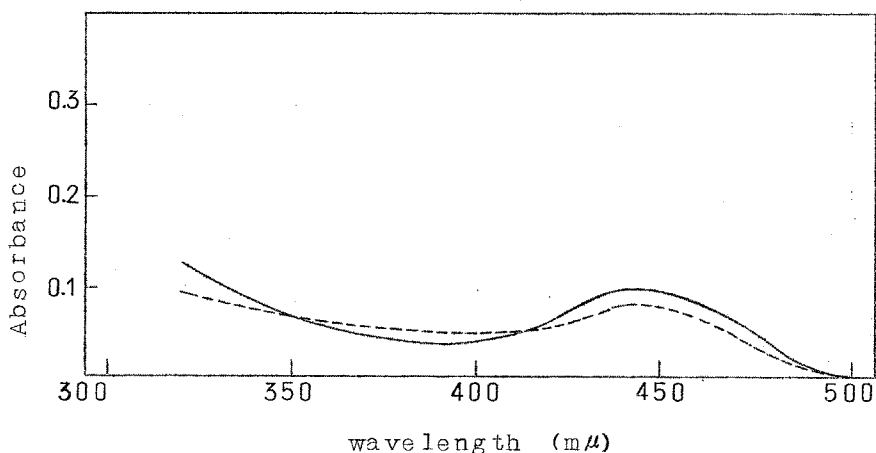


Fig. VI-8 Absorption spectra of Derivatives Modified with CHD.

— CHD-R-I (435 $\mu\text{g/ml}$, at pH 10.5)
---- CHD-R-III (350 $\mu\text{g/ml}$, at pH 10.5)

Table VI-4 Reactive Site Classifications of Various
Inhibitors

Arg - type	Lys - type
R - I	R - II
B - I	R - III
	B - II
STI	B - III

Chapter VII Isolation and Properties of Inhibitor-Enzyme Complex

Since soybean trypsin inhibitor-trypsin complex has been isolated and crystallized by Kunitz,⁴⁷⁾ many naturally occurring trypsin inhibitors were prepared in the state of complexes with trypsin. These complexes were very stable against heat-treatment at acidic pH and the dissociation constants were extensively small, $\sim 10^{-10}$ M. The inhibitors R-I and R-III obtained from R. sativus have the molecular weights of 8,000 and 12,000, respectively and the molecular weight of trypsin is estimated as 24,000. In addition, the dissociation constants of complexes between trypsin and inhibitors are approximately 10^{-10} M. In the light of that, it appears that the gel-filtration is very suitable way to isolate the complex from the mixture of enzyme and inhibitors.

In this Chapter, the isolation and properties of complex are described in order to discuss the nature of the interaction between trypsin and inhibitors.

1) Isolation of Trypsin-Inhibitor Complex

The crystalline trypsin and the purified inhibitor were mixed in sodium acetate buffer, pH 6.0 at the slightly high levels of the amount of inhibitor. After the incubation for 5-10 min at room temperature, the reaction mixture was applied on the column of Sephadex G-75, which was bufferized

with the same buffer. The elution was performed by using a sodium acetate buffer and the determination of complex protein was done by the method as described in following section. The gel-filtration pattern of trypsin-inhibitor R-III complex is shown in Fig. VII-1.

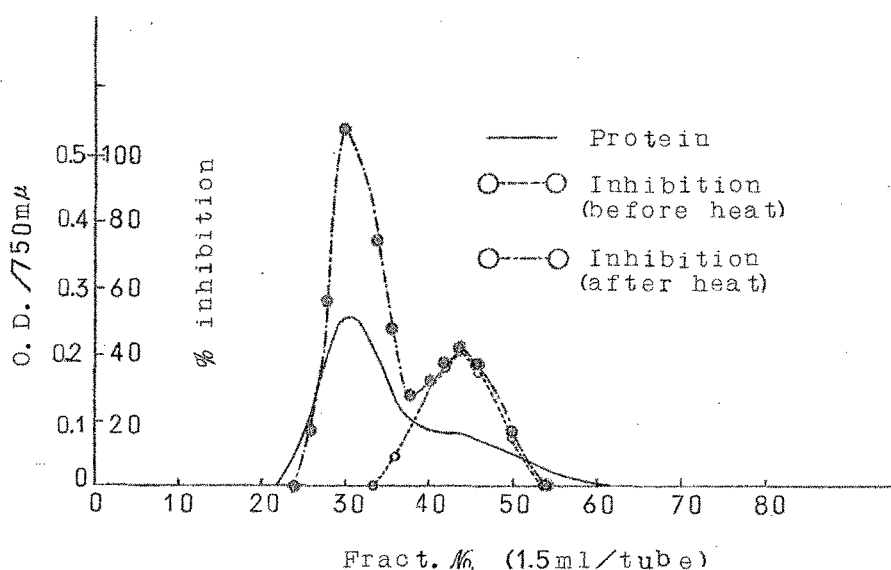


Fig. VII-1 Gel-filtration Pattern of Trypsin-R-III Complex on Sephadex G-75 column. The buffer system used was 0.02 M sodium acetate, pH 6.0. Prior to the application on Sephadex column, 10 mg of R-III and 20 mg of trypsin were incubated for 10 min in 3 ml of buffer solution. 20 μ l of each fraction were used for inhibition assay with the heat activation method described in the text.

2) Determination of the Potential Tryptic or Inhibitory Activity of Complex

The complex at neutral pH has almost no tryptic or inhibitory activity. In order to determine the complex in the chromatographic eluates and the dissociation constants at various pH, two modified procedures were employed.

(i) The inhibitor was more stable than trypsin to the heat treatment at neutral or acidic pH. Therefore, the eluate from gel-filtration was heated at 98°C for 5 min and then the inhibitory activity which was released from the complex in proportion to the inactivation of trypsin was measured.

(ii) The complex partially or fully dissociated at low pH range (pH 2 - 4) and the reaction of trypsin with inhibitor was competitive with substrate, such as BAEE. Therefore, the trypsin freed from the complex can be trapped by substrate when the excess BAEE was added to the trypsin-inhibitor reaction mixture. The excess BAEE solution adjusted to pH 8.0, which contained the trypsin-inhibitor complex, was pipetted into the test cuvet set in the pH-Stat equipment and then the pH value of the solution was reduced to a given acidic pH by the addition of 0.1N-HCl with continuous stirring. After a while, about one minute, the pH of the solution was brought up to pH 8.0 quickly by the addition of 0.1N-NaOH and the activity of trypsin released from the complex was measured. The suitable concentration of the substrate was determined by the displacement assay described in

Chapter VI 2), and 5 mM BAEE at which the displacement was almostly negligible were employed.

3) Electrophoretic Behavior of Complex

The complex of the trypsin and R-III obtained from the gel-filtration on Sephadex G-75 was used in this experiment. The electrophoresis was carried out with the polyacrylamide gel and the cellulose acetate (Oxisoid). Electrophoresis on cellulose acetate strips was carried out in barbital buffer (0.07M, pH 8.6) and a current of 0.7 mA per cm width for 25 min. After development with Ponceau 3R, the complex migrated toward the negative pole and it occurred between trypsin and R-III (Fig. VII-2a). The same results was obtained from the electrophoresis on polyacrylamide gel which was carried out in borate buffer, pH 8.3 and developed with Amino black 10B, (Fig. VII-2b). These results indicate that the complex protein has a pI between that of trypsin and inhibitor and behave as a different characteristic protein.

4) Heat Stability and Effect of Denaturing Reagent

The complex in the buffer solution of sodium acetate, pH 5.5 were placed in a water bath at various temperature, removed at various times, cooled in the ice bath and measured for the appeared activity of trypsin or inhibitor which was released from complex. On the other hand, these procedures

were performed in the presence of the denaturing reagents in order to elucidate the stability, the binding force and nature of complex. When the complex was treated with heat in the absence of salt or denaturing reagent the active inhibitor was appeared in the reaction medium with time whereas the maximum of the activity was less than theoretical value as shown in Fig. VII-3. Similarly, in the presence of denaturing reagents, the active inhibitor was released from the complex faster and greater than in the absence of reagents. (Fig. VII-3)

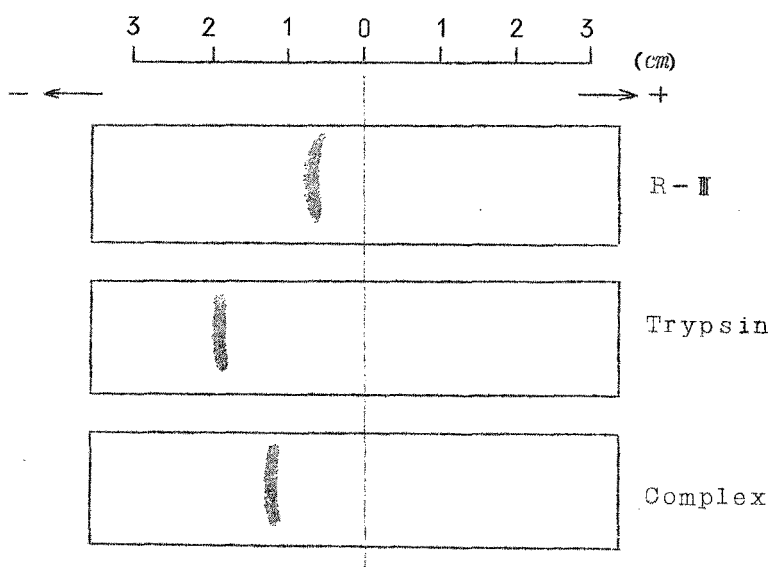


Fig. VII-2a Cellulose Acetate Electrophoresis of Complex, Trypsin and Inhibitor. Electrophoresis was carried out in barbital buffer pH 8.0 using 0.7 mA per cm strip width for 25 min.

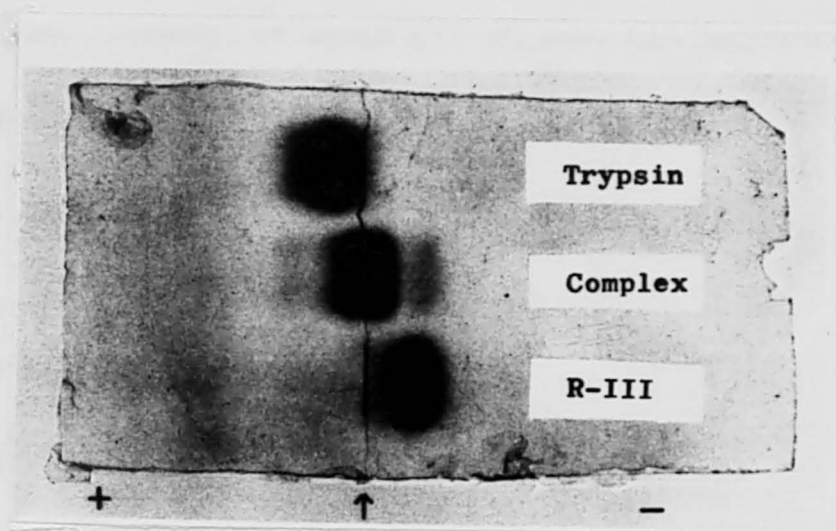


Fig. VII-2b Polyacrylamide Gel Electrophoresis of Trypsin, Complex and R - III.
Electrophoresis was carried out in borate buffer (pH 8.3) 1 mA/cm, 3 hrs.

However, in the case of later experiment, the considerable decrease of activity of the released inhibitor was observed. These result indicate that the release of active inhibitor is due to the inactivation of trypsin, which lost the capacity of binding to inhibitor protein. In the case of urea, as shown in Fig. VII-4, the release of inhibitor was apparently in agreement with the inactivation of free trypsin itself and the decrease of the activity of the released inhibitor corresponded to its own inactivation with urea resulting from the prolonged incubation time. Therefore,

it seemed that the complex formation might not stabilize each component and that the binding of two proteins was comparatively weak to the protein denaturing reagents.

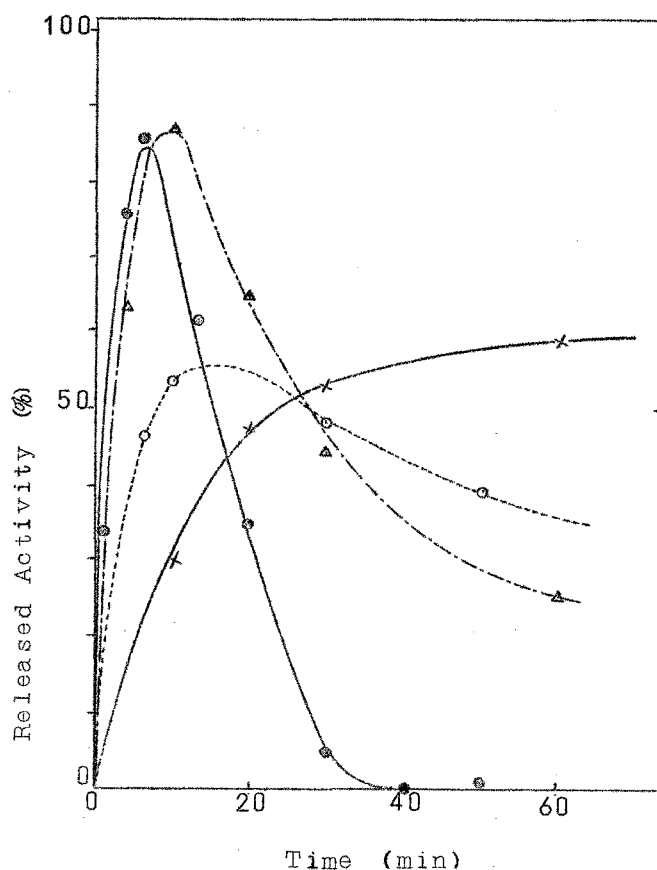


Fig. VII-3 Effect of Heat and Denaturing Reagents on Stability of Complex (Trypsin-R-III)
The experiments were performed in 0.05 M phosphate buffer, pH 7.0 and at 90°C.

x—x—x none
 Δ—Δ—Δ in 4 M urea
 ●—●—● in 30% dioxane
 ○—○—○ in 1.5 M NaCl

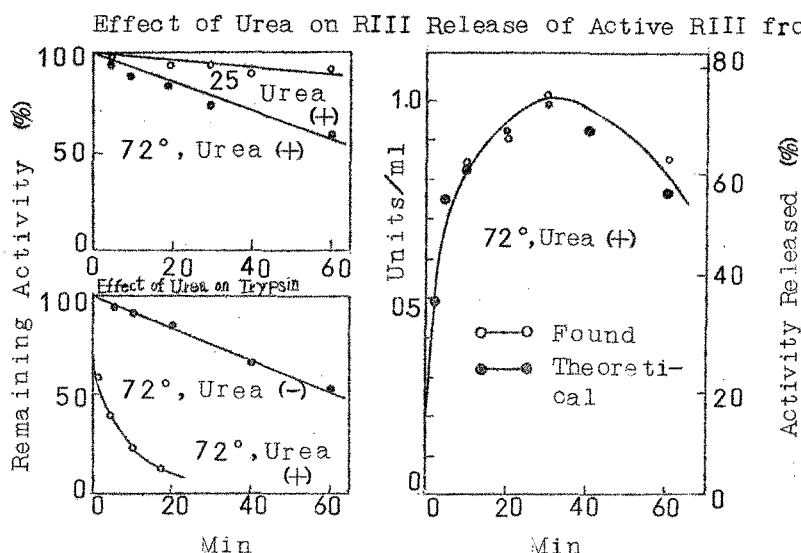


Fig. VII-4 Effect of Urea on Complex

These experiments were performed in 0.05 M phosphate buffer, pH 7.0. For the determination of effect of urea, 200 $\mu\text{g/ml}$ trypsin or inhibitor solution were used. For the measurement of the release of inhibitor, 100 μg of complex in 2 ml of buffer solution were used.

5) Effect of pH on Complex

This experiment was performed according to the procedure as described in section 2), (ii).

The equimolar trypsin and inhibitor, about 10^{-7}M , in 0.2 ml of 0.01M phosphate buffer was incubated and combined at pH 8.0, Two ml of aqueous solution of 5 mM BAEE adjusted to pH 8.0 was added to the complex solution and the pH of this mixture was reduced to a given acidic pH by using

0.1N-HCl with continuous stirring. After standing for one minute, the pH was quickly raised up to 8.0 with 0.1N-NaOH and the activity of free trypsin was measured. The dissociation curves obtained from the plot of the amounts of free trypsin at various pH were shown in Fig. VII-5. The dissociation constants of trypsin and inhibitor complexes apparently depend on pH. The relationship between pH and dissociation constant of trypsin-R-III complex is shown in Fig. VII-6. The complexes used in this experiment dissociated in the acidic pH medium below pH 5.0 and the dissociation curves that depended on pH were found to be typical sigmoid. These observations suggest that for the complex formation there can be the contribution of carboxyl groups that has been pointed out by Green.⁴⁵⁾ According to D'Albis,⁴⁸⁾ in the specific inhibition of trypsin by benzylamine their complex formation was also affected by pH change in acidic medium and the affinity of benzylamine for trypsin was due to the existence of an interaction between the positive charge of the inhibitor and negative charge of a carboxyl-group of the enzyme having pK 4.7. However, the sigmoidal curves of dissociation obtained from the trypsin-protein inhibitor complexes did not seem to conform to first order sigmoid. It suggests that the carboxyl groups contribute not only to binding but to maintenance of protein conformation.

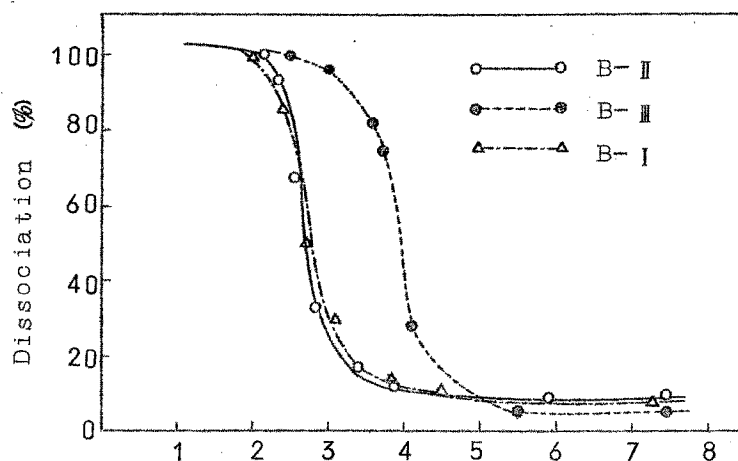


Fig. VII-5 Effect of pH on Complex (1)

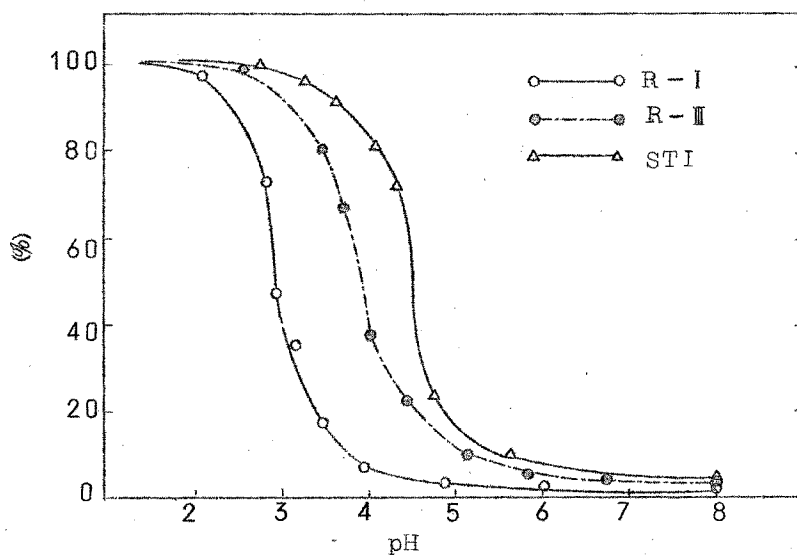


Fig. VII-5 Effect of pH on Complex (2)
 The experimental procedures are described in the text.
 The equimolar quantities of trypsin and inhibitor, about $10^{-7}M$, were used in this assay.

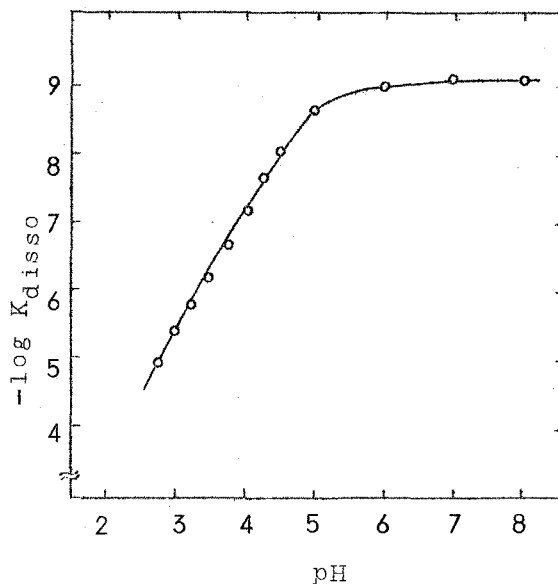


Fig. VII-6 pH Dependence of Dissociation Constant of Trypsin-R-III Complex.

Therefore, the dissociation in acidic medium seems to be caused by not only a discharge of carboxyl groups but also the conformational change - reversible denaturation of these proteins.

Postulating a half dissociating point of complex is designated as pK , the pK values of several complexes are estimated from the dissociation curves as Table VII-1. These values are specific to inhibitors so that it is possible to identify the species of inhibitors by using them.

In addition, the TNP-R-I and TNP-STI which were described in Chapter VI were also examined in comparison with the native

inhibitors. R-I and STI are both arginine type inhibitors which can combine with trypsin in spite of the modification with TNBS. The dissociation curves of the modified inhibitor-trypsin complex are given in Fig. VII-7. It appeared that pK values of these complexes were reduced about 0.5 pH in comparison with the native inhibitor-trypsin complex. It may be considered that the decrease of pK is due to the change of the environment around carboxyl groups caused by the introduction of trinitrophenyl groups resulting the abnormal ionization of carboxyl groups.

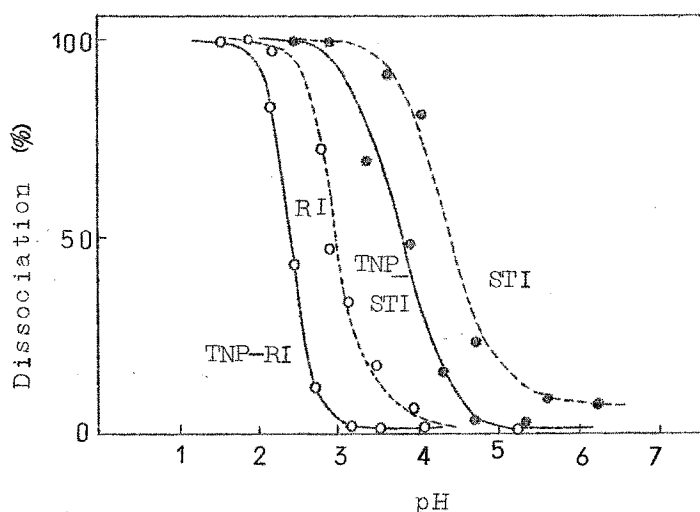


Fig. VII-7 Effect of pH on Complex
Experimental conditions were the same as Fig. VII-5.

Table VII-1 pK Values of Various Complexes:

Complex	T-R-I	T-R-III	T-STI	T-TNP-R-I
pK	2.9	3.8	4.5	2.4

Complex	T-B-I	T-B-II	T-B-III	T-TNP-STI
pK	2.8	2.7	3.9	4.0

* These values were estimated from the results of Fig-VII-5 and Fig. VII-7

Chapter VIII Additional Remarks

1) Location of Inhibitors

Plant protein which inhibits various proteinases, although apparently not ubiquitous in plant, are common in plant storage organs of large number of species; seed, fruit and rhizome.

In the case of seed, especially of dicotyledonous plants, it has not been clear where the inhibitors located. Therefore, the author tried to determine the inhibitor in various parts of seed in order to elucidate its location. Although the extracts from various parts of seed were examined for the inhibitory activity, only the extract of cotyledone gave the inhibition behavior for proteinases. This observation contrasted with the case of barley.¹¹⁾

2) Change of Inhibitor Activity during Germination

Although the inhibitors occurred in only cotyledone, the inhibitory activity disappeared in the course of germination, but not completely (Fig. VIII-1). The similar observation was reported in the case of lettuce seed by Shine et al.¹²⁾ From these results it may be considered that the inhibitor proteins can be actual components of the seed protein which is reserved in cotyledone. However, it is still obscure whether the activity to form complex with proteolytic enzymes related to physiological phenomena in the intact plant. The author made efforts to elucidate the occurrence

of the endogenous proteinases in seed which expected to be inhibited by the endogenous inhibitors, but could not obtain the positive results.

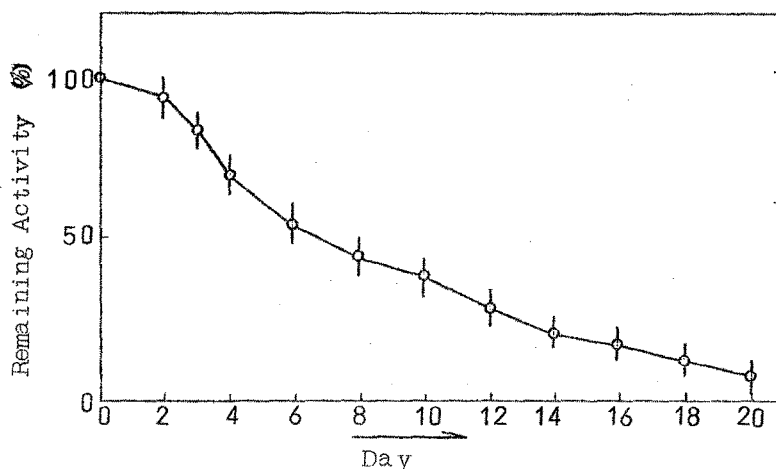


Fig. VIII-1 Change of Inhibitor Activity in Cotyledon during Germination (*R. sativus*)
Lots of 5 g seeds were germinated on vermiculite and a daylight throughout the course of this experiment. Each lot was taken out at indicated periods and determined.

3) Change of Inhibitory Activity during Defatting Proces.

Rape seed is principal source of the vegetable oil in Japan. Therefore, the behavior of inhibitors during the process of defatting was examined. It may be considered important to elucidate the significances of inhibitors in

deffated meal before employing it to the reseach of the chemistry and nutrition of wasted protein.

In the case of B. jucea, as shown in Table VIII-1, the activity still remained after the extraction with expeller, but after the treatment with hexane disappeared. Concidering that the inhibitors are comparatively stable against the heat treatment in acidic medium, it seems the coinsidental fact. The similar observation was also obtained by Rackis⁴⁹⁾ with respect to soybean meal processing. Therefore, it may be considered that the occurrence of these inhibitors are one of the major factors responsible for the poor utilization of proteins in untreated seeds.

Table VIII-1 Change of Inhibitor Activity During Defatting Process

Step	Total N (mg/100mg)	Total Inhibitor unit (unit/mg)	Unit/mg N
Raw Seed	3.53	1056	302
Meal I (Extracted with Expeller)	5.15	936	180
Meal II (Extracted with Hexane)	5.88	0	0

Chapter IX Summary and Conclusion

The author has attempted to elucidate the occurrence of proteinase inhibitors in plant seeds and the relation between the chemical properties and the biological significance of inhibitor proteins. The results obtained in these studies are summarized as follows.

1. (Chapter II) The distribution of proteinase inhibitors in plant seeds were investigated, mainly with respect to Cruciferae. All species used in this study presented the strong inhibitory activity for various proteinases, especially trypsin, for which the inhibition was stoichiometrical and the inhibitor unit was varied from 5,280 of B. oleracea to 1,010 of M. incana. It was found that inhibitors in seeds of Cruciferae were composed of at least three or four components, which differed in isoelectric point and in molecular weight, when the crude extract was separated with the gel-filtration on Sephadex and the iso-electrofocusing.

2. (Chapter III) The preparation and fractionation of inhibitors from the crude extracts of seeds by the use of CM- and SE-cellulose ion exchange were established. From the extract of R. sativus three different types of inhibitors, R-I, R-II and R-III were fractionated and, among them, R-I and R-III could be purified. In the same way, four inhibitor components, B-I, B-II, B-III and B-IV were obtained

from the extract of B. juncea.

3. (Chapter IV) The purified inhibitors obtained from R. sativus gave the following characters as protein (Table IX-1).

Table IX-1 General Properties of R-I, R-III

M. W.	Stoichiometry	8,000	12,000
	Ultracentrifuge	-	11,200
	A.A. analysis	7,100	11,000
$S_{20, w}$		-	0.98 S
$E_{280}^{1\%}$		9.8	9.6
N - terminal		Val.	Val.
U. V.	max.	276 mμ	275 mμ
	min.	250 mμ	254 mμ
p^I		4.6	6.2
N %		-	15.1

Amino acid composition of these proteins were different from each other while the contents of Met, His and Try were very low.

These inhibitors in acidic medium were considerably stable in heating above 80°C.

4. (Chapter V) The inhibition spectra of inhibitors were

determined with various enzyme activities. All showed the inhibition of a polyvalent nature as follows:

Table IX-2 Types and Specificities of Inhibitors

Inhibition	Arg - type			Lys - type			
	R-I	B-I	STI	R-II	R-III	B-II	B-III
Trypsin (casein, BAEE)	+	+	+	+	+	+	+
Pronase (casein, BApNA)	+	+	+	+	+	+	+
Chymotrypsin (BTpNA, casein)	-	+	+	-	+	+	+
Nagarse (ATEE)	+	-	-	-	+	+	+
(casein)	+	-	-	-	-		-
Pepsin (HCl-Hb)	-	-	-	-	-	-	-
Papain (BApNA)	-	-	-	-	-	-	-

+ Inhibit, - Do not inhibit

However, that is, "multi-headed type" character could not be obtained.

5. (Chapter VI) The reaction of inhibitor with trypsin appeared to be competitive type and reversible and to conform to second-order kinetics. The rate constants of the inter-

action between trypsin and inhibitors were approximately calculated as $10^6 \text{ l. mole}^{-1} \text{ sec}^{-1}$. The dissociation constants of these complexes were found to be about 10^{-10} M , at pH 8.0, while they depended on pH. From the results of the rate estimation it was assumed that the mechanism of the interaction between trypsin and inhibitors contained a "mono-molecular" process which would not be affected with the viscosity and ionic strength of the reaction medium.

From the determination of the reacting groups in inhibitor proteins it was concluded that there existed two different types of inhibitors; one of them required an arginine residue in combining with trypsin and the other did a lysine residue. These different types of inhibitors occurred in the same seed.

6. (Chapter VII) Owing to the gel-filtration on Sephadex G-75, the complex protein of trypsin and inhibitors was isolated. In order to determine the inert complex, the improved methods with which the potential inhibitory and triptic activity was measured, were established. The complex protein behaved as a single characteristic protein in the electrophoretic determination on polyacrylamide gel or cellulose acetate strip.

From the results of heat treatment in the presence of protein denaturing reagents, it was found that the complex

formation could not contribute to the stabilization of each component.

Effect of pH on complexes in acidic region gave the typical dissociation curves and pK values, which was meaning a half-dissociating point, were estimated with respect to each species. These values were specific to each inhibitor so that they might be used to identify the species of inhibitors.

7. (Chapter VIII) The inhibitors in seeds located only in cotyledone and their activities were gradually disappeared during germination. However, the inhibitors, in spite of the cotyledonous protein, could not be clarify their significant role in the intact plant. These important problems were left behind in the field of plant physiology.

ACKNOWLEDGEMENT

The author wishes to express his sincere gratitude to Dr. Tadao Hata, Professor of Kyoto University, for his kind guidance and encouragement. The author is also grateful to Dr. Etsushiro Doi, Assistant Professor of Kyoto University, for his kindful advices and suggestion throughout the course of this work.

The author is greatly indepted to Mr. Rikimaru Hayashi and Mr. Takahiko Higasa for their constant cooperations and discussions.

Thanks are given to the members of the Research Institute for Food Science, Kyoto University, for their helpful discussion in carrying out this work.

The author wish to express his thanks to Mr. Y. Takii, Takii and Co., Ltd., for the supply of many kinds of seeds.

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